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From the Pharmacological Department, University of Lund, Sweden.

The Effect of Vitamin D on the Absorption of Inorganic Phosphate.

By

ARVID CARLSSON.

With the technical assistance of

M. LINDQVIST and T. MAGNUSSON.

Received 4 January 1954.

Numerous balance experiments have shown that the fecal excretion of both Ca and P may be increased in vitamin D deficiency. The mechanism of this disturbance was studied in detail by NICOLAYSEN (1937 a and b). The addition of Ca to a basal diet that was essentially free of both Ca and P was found in rats to produce an increased excretion not only of Ca but also of P in the feces. On the other hand, when P was added instead of Ca it seemed to be completely absorbed, whether it was given as inorganic phosphate, glycerophosphate, or casein, the fecal Ca and P being unaffected. In both types of experiments, and even if the basal diet was given without any addition, vitamin D was found to reduce the amounts or Ca and P in the feces. It was concluded that vitamin D affected primarily the absorption of Ca (including the reabsorption of endogenous Ca), the action on the absorption of P being indirect: when the amount of Ca in the intestinal tract was reduced as a consequence of increased absorption, less Ca phosphate was precipitated, and more phosphate was thus absorbed.

There are certain difficulties in this interpretation. When the fecal P is plotted against the fecal Ca, the curve of the vitamin D treated animals is found to lie definitely below that of the non-treated animals (Figure 1; the data are averages taken from Tables I and II, NICOLAYSEN 1937 a; values from animals said

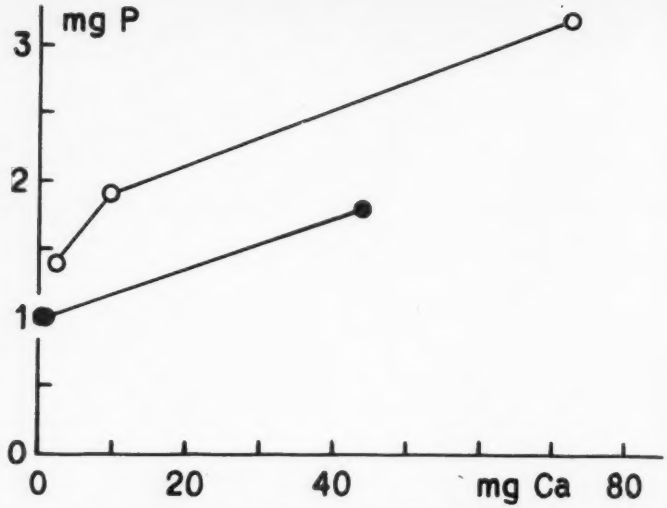

Fig. 1. The Daily Excretion of P in Relation to the Daily Excretion of Ca in the Feces of Vitamin D Deficient Rats (Circles) and Rats Treated with Vitamin D (Dots).
The 3 values of either curve were obtained on Ca intakes of 2.3 mg, 15 mg, and 90 mg daily. The data are taken from NICOLAYSEN (1937 a).

to be in an early stage of vitamin D deficiency were omitted, since in these animals there was no effect of vitamin D on the fecal P). The conclusion that the influence of vitamin D on the absorption of P is referable to a reduced amount of Ca in the intestinal tract therefore seems hardly justified.

COHN and GREENBERG (1939) reported that in rats the absorption of P^{32}, given as inorganic phosphate, was increased but little by vitamin D. The tracer was given 1 hour after the vitamin. At this short interval an effect on the absorption can hardly be expected (compare LINDQUIST 1952).

Below some experiments are given in which the action of vitamin D on the absorption of inorganic phosphate is further studied.

Experimental.

Two experimental series with different diets were studied. In the first series the Ca "free" diet described in an earlier paper (CARLSSON 1952) was used. It contained 0.04 per cent Ca and 0.5 per cent P. In the second series $CaCO_3$ had been added to this diet so that the Ca

content was 2.0 per cent. The growth of animals kept on the former diet is increased by vitamin D, whereas on the latter diet there is no such effect, the daily increase in body weight being about 2 grams both in the presence and in the absence of vitamin D.

Three-week-old rats were transferred to either of these diets. After another 3 weeks in the first series and 5 weeks in the second series Ca^{45} and P^{32} were given. About half of the animals in either series received calciferol. In the first series some of the animals were given 2 doses of calciferol, viz. 11 and 3 days, respectively, before the administration of the isotopes. The remaining animals in the first series and all the animals in the second series received a single dose of calciferol 3 days before the isotopes were given. Each dose contained 100 I. U. of vitamin D, given orally in 0.1 ml peanut oil. The values obtained for Ca^{45} and P^{32} absorption were practically the same whether 1 or 2 doses of calciferol were given. In the second series about half of the animals were parathyroidectomized (Richter and Birmingham 1941) 16 days before the administration of the isotopes. The remaining animals in the series were sham-operated, *i. e.*, the parathyroids were exposed but not removed.

The isotopes were administered in doses of a few microcuries. Solutions containing Ca^{45} in Ca lactate and P^{32} in $Na_3\,PO_4$ were mixed. The freshly prepared mixture containing a fine precipitate of Ca phosphate was given by stomach tube. The dose of P was 2.5 mg throughout. In the first series Ca was given in a dose of 5 mg (which is almost equivalent to 2.5 mg P), 10 mg, or 20 mg. In the second series all the animals received 5 mg Ca. The volume of fluid administered was 1.5 ml.

The urine and feces of each animal were collected separately. The rats were killed 18 hours after the isotopes were given. The Ca^{45} and P^{32} in the gastrointestinal tract + feces were estimated. The percentage absorption of either isotope was said to be 100 — (per cent of dose in gastrointestinal tract + feces). For further technical details, see Carlsson (1951, 1952).

Results.

The results of the first experimental series, in which the animals were kept on the Ca "free" diet, are given in Figure 2. The *percentage* absorption of Ca was fairly independent of the amount of Ca administered, in agreement with earlier findings (Carlsson 1951); it was almost doubled after vitamin D administration. The absorption of P, which was given in a dose of 2.5 mg throughout, was but slightly affected by the amount of Ca administered at the same time. It was increased by vitamin D, although somewhat less than the Ca absorption. The increase in P absorption produced by vitamin D could not be attributed to a reduced

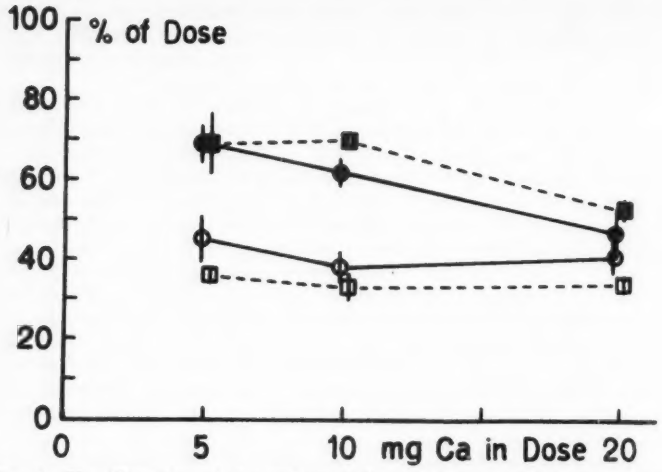

Fig. 2. The Absorption of Ca^{45} and P^{32} at Varying Dosage of Ca in Rats Kept on a Ca "Free" Diet.
Squares: Ca^{45}. Circles: P^{32}. Open symbols: vitamin D deficient animals. Filled-in symbols: vitamin D treated animals.
Each symbol indicates mean ± standard error of the mean.

	Nr of rats given the Ca dose of		
Vitamin D	5 mg	10 mg	20 mg
—	5	11	9
+	5	10	10

amount of Ca in the intestine. This will be apparent from Figure 3, where the amount of non-absorbed P is plotted against the amount of non-absorbed Ca. Vitamin D treatment resulted in a lower amount of non-absorbed P even when the difference in non-absorbed Ca was accounted for. The data seem to be in fairly good agreement with those of NICOLAYSEN given in Figure 1.

Table 1 gives the results of the second series, in which the animals were kept on a diet with a high Ca/P ratio. The test dose was the same in all the animals and contained 5 mg Ca and 2.5 mg P. In part of the animals the parathyroids had been removed 16 days earlier, which had no detectable influence on the absorption of Ca and P. The absorption of Ca was markedly increased by vitamin D, but there was no effect on the absorption of P.

Discussion.

The influence of vitamin D on the absorption of Ca was appreciable in both experimental series, but an effect on the absorption

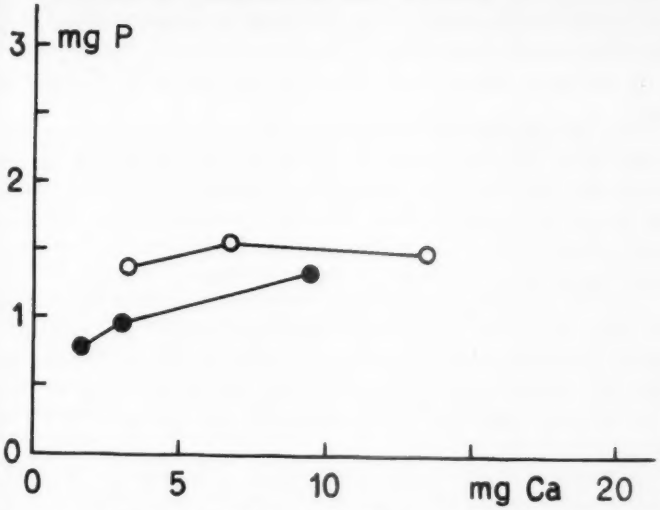

Fig. 3. Non-Absorbed P in Relation to Non-Absorbed Ca in Vitamin D Deficient Rats (Circles) and Vitamin D Treated Rats (Dots).
The values were calculated from the data in Figure 2.

Table 1.

The Effect of Vitamin D on the Absorption of Ca^{45} and P^{32} in Rats Kept on a Diet with a High Ca/P Ratio.

The values are expressed in per cent of the administered dose.

Vitamin D	Parathyroids present			Parathyroids removed		
	Nr. of rats	Ca^{45}	P^{32}	Nr. of rats	Ca^{45}	P^{32}
—	7	25	51	8	18	46
+	8	38	49	8	43	52
Difference		13	— 2		25	6
± standard error		4.0	7.1		3.8	6.3
P %		< 1	> 50		< 0.1	> 25

of P was detectable only in the first series. Under the conditions of the first experimental series, in which the animals were deficient in Ca, vitamin D exerts a marked effect on the growth and general well-being of the animals, presumably by restoring the lowered serum Ca to normal (CARLSSON 1952). We have observed no similar action in animals kept on the diet with a high Ca/P

ratio used in the second series. It therefore seems probable that the effect on the absorption of P seen in the first series is indirect and related in some way to the action on the growth of the animals.

The observations of NICOLAYSEN (l. c.) seem to be in agreement with this interpretation, as far as can be judged by the available data. The rats used in his experiments gained in body weight about 1 gram daily. This gain was regular in the rats given vitamin D, whereas the vitamin D deficient rats stopped growing after 4 to 7 weeks. In the early stage of vitamin D deficiency the fecal excretion of P was not always affected by vitamin D, while in the late stage the effect was apparent in all the experiments. Thus even here the influence of the vitamin on the absorption of P seemed to be related to the growth of the animals. As in the present experiments the absorption of Ca was invariably improved by the vitamin.

The experiments so far discussed do not exclude the possibility that the concentration of Ca ions in the intestinal tract plays an important rôle for the absorption of the phosphate of an ester such as phytin, the Ca salt of which has an extremely low solubility. Indeed this is indicated by the work of DAY (1940), in which the intestinal concentration of Ca ions was varied by adding citrate to the diet. In accordance with the present findings citrate addition was without influence on the absorption of P, when the diet contained no phytin, whereas the effect was marked, when phytin-containing diets were used. It therefore seems probable that the effect of vitamin D on the absorption of P, as observed in ordinary balance experiments by *e. g.* ALBRIGHT and SULKOWITCH (1938), may be at least partly referable to a reduced precipitation of Ca phytate resulting from an increased absorption of Ca.

Summary.

The absorption of P^{32} given orally as inorganic phosphate together with varying amounts of Ca was studied in vitamin D deficient rats and in rats treated with this vitamin.

In Ca deficient animals vitamin D increased the absorption of phosphate. The effect was probably indirect and related to an improvement of the general condition of the animals. In rats kept on a diet with a high Ca/P ratio vitamin D had no influence on

the absorption of phosphate. In both instances the absorption of Ca was increased by the vitamin.

The absorption of inorganic phosphate was found to be fairly independent of the amount of Ca in the intestinal tract.

This investigation was aided by grants from the Swedish State Medical Research Council.

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From the Pharmacological Department, University of Lund, Sweden.

The Cause of Hypophosphatemia and Hypocalcemia in Vitamin D Deficiency.

By

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With the technical assistance of

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A distinctive finding in vitamin D deficiency is a decrease in the product Ca × P of the serum. The phenomenon is generally considered to be the consequence of poor absorption of the elements from the intestinal tract. In addition, an increased excretion of P in the urine, owing to stimulation of the parathyroids, has been claimed to contribute to the lowered P level (for references, see ALBRIGHT and REIFENSTEIN 1948).

In an earlier paper (CARLSSON 1952) we reported that the ability of vitamin D to maintain a normal level of serum Ca persisted in the virtual absence of food Ca. Evidence was presented that under these conditions the vitamin acted by promoting the solution of bone salt.

In the present paper it is demonstrated that the effect of vitamin D on the concentration of serum P persists under conditions in which the action on the absorption and excretion of the element is negligible. Further evidence is provided that the vitamin D deficient animal cannot mobilize its bone stores.

Experimental.

Three-week-old rats were transferred to a diet containing no vitamin D but adequate amounts of Ca and P (0.8 and 0.5 per cent, respectively). The diet was prepared by adding $CaCO_3$ to the Ca "free" diet described in an earlier paper (CARLSSON 1952). At the age of 9 weeks the animals

were deprived of their diet and were instead given a sugar-salt mixture consisting of 93 per cent sucrose, 1 per cent sodium chloride, and 6 per cent Ca lactate. The mixture was found by analysis to be essentially free of P. It contained 0.8 per cent Ca. After 24 hours on this mixture half of the animals received 100 I. U. calciferol in 0.1 ml peanut oil. After another 3 days each animal was given by stomach tube 1 ml of a solution containing a few microcuries of P^{32} of a high specific activity and 0.3 grams of the sugar-salt mixture. Urine and feces were collected separately. The animals were bled under ether anesthesia 24 hours after the administration of this solution. The following estimations were made: Ca and (inorganic) P in the serum, P^{32} in feces + contents of the gastrointestinal tract, in the urine, in both tibia-fibulae, and in the 4 incisors. For further technical details, see CARLSSON (1951, 1952).

During the period on the sugar-salt mixture the animals lost in weight about 3 grams daily but otherwise showed no obvious signs of abnormality.

Another experiment was performed in order to study the ability of vitamin D deficient rats to utilize their bone stores. Seventy-day-old rats which after weaning had been fed on the diet with a good Ca/P ratio described above, were divided into 3 groups of 5 animals. One group was kept as control, while the other 2 groups were transferred to the Ca "free" diet mentioned above. One of these groups had been given 100 I. U. of vitamin D 3 days earlier. Twenty-four hours after the 2 groups had been placed on the Ca "free" diet all the animals were bled under ether anesthesia, and the serum contents of Ca and P were estimated.

Results and Discussion.

In the animals on the P free diet vitamin D produced a marked increase in the serum P level, from 3.6 to 5.6 mg per 100 ml (Table 1). The administered P^{32} was absorbed almost completely in both groups. Presumably the reabsorption of the P that entered the lumen of the gastrointestinal tract as a constituent of the various digestive juices, was equally complete. The amount of P^{32} recovered from the urine was insignificant, and no difference between the 2 groups was detectable. The vitamin caused an increased uptake of P^{32} in the tibia-fibulae, while the P^{32} of the incisors showed a statistically almost significant decrease.

Thus vitamin D produced its usual effects on the serum P level and on the skeletal uptake of P^{32} even in animals which had no P in their food and which were able to retain their P almost completely.

In the other experiment the ability of vitamin D deficient animals to utilize their bone stores was studied (Table 2). The

Table 1.

Effect of Vitamin D on P^{32} Metabolism and on Serum Ca and P in the Absence of Dietary P.

	Vit. D		Difference ± S. E.	P %
	—	+		
P^{32} % dose				
Absorption [1]	94.3	96.3	2.0 ± 0.54	< 0.5
Urine	0.2	0.2	0.0	—
Tibia-fibulae	2.9	3.8	0.9 ± 0.13	< 0.1
Incisors	2.0	1.7	— 0.3 ± 0.11	< 2.5
Serum, mg per 100 ml				
Ca	11.1	11.7	0.6 ± 0.38	> 10
P	3.6	5.6	2.0 ± 0.34	< 0.1
Nr of rats	8	9		

[1] 100 — (% dose in gastrointestinal contents + feces).

Table 2.

Effect of Ca Starvation for 24 Hours on Serum Ca and P.

Ca in Diet	Vit. D	Nr of Rats	Serum, mg per 100 ml	
			Ca[1]	P
—	+	5	9.4	8.7
—	—	5	6.9	8.7
+	—	5	8.0	7.3

[1] The difference between 2 groups of animals which was necessary for P = 1 per cent, was found by analysis of variance to be 1.3 mg per 100 ml.

animals had been kept for several weeks on a diet with a good Ca/P ratio, and their bone stores were thus well filled; the ash of their left tibia-fibula weighed 76 mg per 100 g body weight, which is only slightly below optimum for the age in question. As is well known the Ca retention of vitamin D deficient rats may be almost normal when they are kept on a diet adequate in Ca and P. It was found that the animals were not able to utilize their bone stores. After 24 hours on a Ca "free" diet they had hypocalcemia, the serum value being 6.9 mg Ca per 100 ml. As shown in Table 2 the effect of Ca starvation could be prevented by vitamin D. In experiments described earlier (CARLSSON 1952) 3 weeks of Ca starvation failed to produce hypocalcemia in animals treated with vitamin D.

The serum Ca of the control animals which remained on the Ca rich diet throughout the experiment but received no vitamin D was 8.0 mg per 100 ml, *i. e.* below the normal range. Thus a Ca absorption sufficiently high to keep the bone stores filled is not very effective in maintaining a normal serum Ca.

It may be concluded that the chief cause of hypophosphatemia and hypocalcemia in vitamin D deficiency is an insufficient utilization of stored bone salt. Reduced absorption of Ca and P may be regarded as contributory rather than essential factors. It may be objected that the absorption may be of greater importance in other species than rats. This does not seem very probable, since the ability of the rat to absorb Ca is generally considered to be very high (NICOLAYSEN and NJAA 1951). Further, the experiments of MELLANBY (1949) do not indicate that the intestinal effect of vitamin D plays a dominating rôle in dogs.

Summary.

In rats on a P free diet vitamin D produced its typical effects on the level of serum inorganic P and on the uptake of P^{32} in the skeleton. These effects could not be explained as consequences of increased absorption or decreased excretion of P, since the element was retained almost completely even in the absence of vitamin D.

Vitamin D deficient rats were found to be unable to utilize their bone stores for maintaining a normal serum Ca, even if the bone stores had been well filled by feeding a diet with a good Ca/P ratio.

It is concluded that the essential cause of hypophosphatemia and hypocalcemia in vitamin D deficiency is an insufficient utilization of stored bone salt.

This investigation was aided by grants from the Swedish State Medical Research Council.

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From the Pharmacological Department, University of Lund, Sweden.

The Influence of Vitamin D on the Uptake of S^{35}-Labelled Sulfate in the Bones.

By

ARVID CARLSSON.

With the technical assistance of

M. LINDQVIST and T. MAGNUSSON.

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After the administration of inorganic sulfate labelled with S^{35} part of the isotope is incorporated into various organic compounds, *e. g.* chondroitin sulfate (DZIEWIATKOWSKI, BENESCH and BENESCH 1949, DZIEWIATKOWSKI 1951, BOSTRÖM 1952). *In-vitro* experiments with slices of cartilage indicate that this incorporation requires the presence of enzymes (BOSTRÖM and MÅNSSON 1952).

There is some evidence that chondroitin sulfate plays an important rôle in calcification (RUBIN and HOWARD 1950, DZIEWIATKOWSKI 1952). It was therefore thought worth while investigating the action of vitamin D on the sulfate metabolism in the bones.

Experimental.

Three diets were employed, with Ca contents of 0.04 per cent, 0.8 per cent, and 2.0 per cent, respectively. The 2 last-mentioned diets were prepared by adding $CaCO_3$ to the first-mentioned diet, which was described in a previous paper (CARLSSON 1952). The P content was 0.5 per cent.

Two-month-old rats were used. Calciferol was administered 3 or 4 days before the injection of S^{35}. It was given orally in a dose of 100 I. U. in 0.1 ml peanut oil. The S^{35}, obtained from A. E. R. E., Harwell, England, in the form of carrier-free inorganic sulfate, was injected intraperitoneally in a dose of 50 microcuries. The urine and the feces

were collected separately. The animals were killed after 16 hours, when the activity of the serum was found to be negligible compared to the activity of the bones. The activity of the bone tissue was slight compared to that of the epiphyseal cartilage. The whole bones, without any separation, were therefore employed. Each bone sample contained both tibia-fibulae and both femora of one animal. The bone samples were treated with 6 N HCl on a boiling water bath for 4 hours and were then diluted to 5 ml. The activities of 2 ml samples were estimated as described in an earlier paper (CARLSSON 1951). The activity of the urine was estimated in the same way after acidification and dilution to 50 ml.

Results.

The influence of vitamin D on the uptake of S^{35} in the bones appeared to depend on the Ca content of the diet (Table 1). An increased uptake was produced by the vitamin, when the diet contained 0.04 per cent or 0.8 per cent Ca. With 2.0 per cent Ca in the diet no effect was observed.

Table 1.

Effect of Vitamin D on the Uptake of S^{35} in the Bones of Rats Kept on Diets with Varying Ca Contents.[1]

	Ca in diet %	Vit. D	Nr of rats	S^{35} % dose		Change in body wt grams
				Bones	Urine	
	0.04	—	5	0.57	67.2	−1
	0.04	+	5	1.04	54.6	+ 9
Difference				0.47	−12.6	10
± standard error				0.100	6.85	1.7
P %				<0.5	>10	<0.1
	0.8	—	4	0.75	74.3	+ 9
	0.8	+	5	1.11	64.0	+13
Difference				0.36	−10.3	4
± standard error				0.085	6.20	2.6
P %				<0.5	>10	>10
	2.0	—	5	0.90	—	+16
	2.0	+	5	0.83	—	+12
Difference				−0.07	—	−4
± standard error				0.11	—	2.2
P %				>10	—	>10

[1] The vitamin was given 3 days before the injection of S^{35} in the experiment with 0.04 per cent Ca in the diet. In the other experiments this interval was 4 days. The change in body weight refers to these 3 and 4 days, respectively.

With 0.04 per cent Ca in the diet vitamin D causes a rise in the serum Ca (cp. CARLSSON 1952). Increasing the Ca content of the diet has a similar action. These two kinds of treatment appeared to be equally effective in stimulating the uptake of S^{35} in the bones and also in promoting the growth of the animals (Table 2).

Table 2.

Effect of Vitamin D on the Uptake of S^{35} in the Bones, as Compared to the Influence of Increased Ca Intake.[1]

	Ca in diet %	Vit. D	Nr of rats	S^{35} % dose		Change in body wt grams
				Bones	Urine	
I	0.04	—	7	0.63	72.6	+ 2
II	0.04	+	7	1.00	70.4	+ 9
III	0.8	—	6	0.98	61.1	+11
Difference II—I				0.37	—2.2	7
± standard error ...				0.165	5.57	1.6
P %				<5	>10	<0.5
Difference III—I ...				0.35	11.5	9
± standard error ...				0.054	6.41	1.6
P %				<0.1	>10	<0.1

[1] The rats in group III were transferred to the diet containing 0.8 per cent Ca at the same time as the rats in group II received vitamin D, *i. e.* 3 days before the injection of S^{35}. The change in body weight refers to this interval.

Table 3.

Effect of Vitamin D on the Uptake of S^{35} in the Bones of Rats on a Restricted Food Intake.[1]

	Ca in diet %	Vit. D	Nr of rats	S^{35} % dose Bones	Change in body wt grams
	0.8	—	5	0.65	—5
	0.8	+	5	0.66	—4
Difference				0.01	1
± standard error				0.083	2.8
P %				>10	>10

[1] The vitamin was given 4 days before the injection of S^{35}. During this interval the consumption of food was 40 grams per rat in both groups of animals. The change in body weight refers to these 4 days.

The difference observed in Table 1 between vitamin D treated and non-treated animals on the diet containing 0.8 per cent Ca, was no longer seen when the consumption of food was made equal in the 2 groups of animals (Table 3). The food intake was made equal by restricting the daily rations, so that they were eaten quantitatively.

Vitamin D seemed to have no significant action on the excretion of S^{35} in the urine.

Discussion.

The present observations indicate that vitamin D has no direct action on the uptake of S^{35} in the bones. The stimulating influence on the S^{35} uptake observed in some of the experiments seemed to be related to the action of the vitamin on the growth of the animals. When growth was promoted by other means than by vitamin D, as in the experiment shown in Table 2, an effect on the uptake of S^{35} in the bones comparable to that of vitamin D was noted. When vitamin D did not increase the rate of growth of the animals, as in the experiment with 2.0 per cent Ca in the diet (Table 1), or in that with the restricted food intake (Table 3), the uptake of S^{35} in the bones was unaffected.

The results fit in with the view that vitamin D has no direct influence on the matrix formation and that the direct action of vitamin D on the bone metabolism is confined to its catabolic phase (cp. CARLSSON 1952).

Summary.

Vitamin D was found to stimulate the uptake of S^{35} in the bones after the injection of S^{35}-labelled sulfate to young rats, but the effect was observed only when the growth of the animals was promoted by the vitamin, and it was therefore considered to be indirect.

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The Effect of Vitamin D on the Citric Acid Metabolism.

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Earlier work in our laboratory showed that one of the fundamental physiological actions of vitamin D is to facilitate the solution of bone salt (CARLSSON 1952). The question arose whether this effect could be related to the influence of the vitamin on the citric acid metabolism. DICKENS (1941) and NICOLAYSEN and NORDBÖ (1943; cp. WAASJÖ and EEG-LARSEN 1951) observed that the citric acid content of the bones was decreased in vitamin D deficiency. FREEMAN and CHANG (1950) found that excessive doses of vitamin D increased the serum citric acid in dogs. This observation was confirmed by HARRISON and HARRISON (1952) in a study on rachitic infants, in which the serum citric acid was also found to be below the normal range. There were parallel changes in the urinary citric acid. In rats BELLIN and STEENBOCK (1952) observed an increased excretion of citric acid in the urine following physiological or excessive doses of vitamin D.

Whether the rise in bone citric acid is due to a local action of vitamin D or to the elevated serum citric acid, is not known; so far, these two effects of vitamin D have not been studied by one and the same investigator. The main part of the bone citric acid is intimately combined with the bone salt and is presumably situated on the surface of the apatite crystals (HENDRICKS and

HILL 1951). The composition of the bone salt is not constant but varies to a certain extent with that of the surrounding fluid, and it is thus influenced not only by local factors but also by the composition of the blood (KUYPER 1945, SOBEL, ROCKENMACHER and KRAMER 1945).

The increase in the concentration of serum citric acid has been found to coincide with other manifestations of vitamin D action, *i. e.*, rise in the levels of serum Ca and P and healing of the rachitic bone lesions (FREEMAN and CHANG l. c., HARRISON and HARRISON l. c.). It has been discussed whether the rise in the serum citric acid is a consequence of the increased serum Ca level. ALWALL (1945) observed that intravenous injection of Ca lactate to rabbits produced an increase in the serum citric acid. Working with dogs CHANG and FREEMAN (1950) failed to detect such an effect; they pointed out that their experiments did not exclude the possibility that a more prolonged hypercalcemia, such as that following vitamin D administration, caused an increase in the level of serum citric acid. In view of the close parallelism between changes in Ca and citric acid produced by vitamin D they were inclined to believe that its mode of action entailed a mechanism affecting both substances; the simplest explanation of this action would be an increase in the liberation of Ca and citric acid from their osseous reservoir. In vitamin D deficient infants HARRISON and HARRISON (l. c.) did not observe any correlation between the levels of serum Ca and citric acid. They therefore suggested that the increased concentration of serum citric acid following vitamin D was not the result of increase in the level of serum Ca but that both of these changes were manifestations of vitamin D effect. As regards the relationship between the levels of serum P and citric acid they arrived at a similar conclusion.

Like vitamin D the parathyroid hormone induces parallel changes in the levels of serum Ca and citric acid (ALWALL 1944). The action of vitamin D on these serum constituents is not mediated by the parathyroids, since it persists in parathyroidectomized dogs (FREEMAN and CHANG l. c.).

The present work was made in order to investigate the effects of physiological doses of vitamin D on the citric acid concentrations in serum and calcified tissues and the causal relationship between these effects and the influence of the vitamin on the mineral metabolism.

Experimental.

As a basal diet we used the Ca "free" diet described in an earlier paper (CARLSSON 1952). It contained 0.04 per cent Ca and 0.5 per cent P. The Ca and P contents were varied by adding $CaCO_3$ and NaH_2PO_4, 2 H_2O, respectively. Three types of diets were used, *i. e.*, the Ca "free" basal diet without any addition; a diet producing P deficiency (2 per cent Ca and 0.5 per cent P); diets containing adequate amounts of Ca and P and with a good Ca/P ratio (0.8 per cent Ca and 0.5 per cent P; or 2.0 per cent Ca and 1.5 per cent P).

Young rats were weaned at the age of 3 weeks and were fed on one of the experimental diets for another 3 weeks. The final age of the animals was thus 6 weeks in all the experiments. In the experiments presented in Figures 1 to 4 the animals were given a single physiological (100 I.U.; Figures 1, 3 and 4) or excessive (100,000 I.U.; Fig. 2) dose of calciferol in 0.1 ml peanut oil orally, controls receiving oil only. The oil, with or without vitamin D, was given at varying intervals before the animals were killed. In each experiment all the animals were sacrificed at the same time.

The results recorded in Figures 4 and 5 were obtained in one single experimental series. The Ca "free" diet was used. Half of the animals received vitamin D at varying intervals as described above (Fig. 4), while at the same intervals their litter mates, instead of having vitamin D, were transferred to a diet containing an adequate amount of Ca (Fig. 5). In this way Ca addition was compared to vitamin D administration.

As to details of these and other experiments, see Figures 1 to 5 and Tables 1 to 4.

Care was always taken to get the different groups of each experiment as equal as possible as regards litter, sex, and body weight.

In all the experiments the animals were killed by bleeding under ether anesthesia. In the blood serum the concentrations of Ca, P (inorganic), and citric acid were determined, and in the left tibia-fibula and the 4 incisors, Ca and citric acid. The citric acid/Ca ratios in the calcified tissues were calculated.

Since the main purpose of the experiments recorded in the Figures was to compare the rates at which different effects of vitamin D developed, the results are expressed in relative values, all the initial values (= the values of the control animals) being set to 100. Obviously this mode of expressing the data facilitates the comparison between different curves.

Ca and P were estimated by the usual oxidimetric and colorimetric methods.

The estimation of citric acid. We used a new modification of the method of PUCHER, SHERMAN and VICKERY (1936). The essential new point in our modification refers to the critical decolorisation step. A variety of reducing agents has been suggested, but all of them are

liable to interfere with the recovery. At the present time most workers seem to prefer hydrogen peroxide (NATELSON, LUGOVOY and PINCUS 1948, BUFFA and PETERS 1950, LARDY 1951, EITINGER, GOLDBAUM and SMITH 1952), but as will be apparent from the paper of *e. g.* BUFFA and PETERS (l. c.), great care must be taken to avoid excess reagent, and the procedure must be performed at reduced temperature. As is well known certain benzene derivatives are easily brominated, and we have utilized this property to remove the bromine. Of several tried substances m-hydroxybenzoic acid was found the most suitable. It can be used in great excess without interfering with the colour development. The MnO_2 and $KMnO_4$ must first be reduced, which is accomplished by the addition of KBr; also this reagent can be added in excess without any interference.

The actual procedure is as follows. As to the serum, a trichloracetic acid extract is prepared in the usual manner. The calcified tissues are split and extracted overnight with 3 ml 30 per cent trichloracetic acid. A suitable aliquot of the extract is transferred to a 50 ml glass-stoppered test tube and diluted with water to 10 ml. The solution is thoroughly mixed with 3 ml 18 N H_2SO_4 (compare KREBS and EGGLESTON 1944), whereupon, in order, 0.5 ml N KBr and 1 ml N $KMnO_4$ are added. The tube is shaken and the mixture allowed to stand for 10 to 15 minutes. About 0.5 ml of the KBr solution is then added. After the last traces of MnO_2 have disappeared, about 2 ml of a saturated solution of m-hydroxybenzoic acid is added and the tubes are shaken until all the bromine is removed. Thirteen ml heptane (refined after NATELSON et al., l. c.) is added, and the tube is shaken vigorously for at least 30 seconds. After the two layers have separated, an aliquot of the heptane layer is transferred to another glass-stoppered test tube, containing 3.5 ml of a 1.5 per cent Na_2S solution in a mixture of 8 volumes of ethylene glycol and 2 volumes of water (compare HUNTER and LELOIR 1945), and the tube is shaken for at least 15 seconds. The bottom layer is transferred to a centrifuge tube and is centrifuged in order to make the solution optically clear. The colour is read at 450 millimicrons in a Beckman spectrophotometer against a blank carried through all the above-mentioned steps. A suitable range is 10 to 80 micrograms citric acid.

Results.

Vitamin D caused an increased level of serum citric acid in all the experimental groups of animals, which were kept on diets with varying Ca and P contents (Figures 1 to 4). There was also a rise in the citric acid content of the bones. In the animals with a good Ca/P ratio in their diet the first-mentioned effect was considerable as early as 24 hours following vitamin D administration, while the latter effect was slight, if detectable at all, at this interval (Figures 1 and 2). In the animals deficient in either P (Fig. 3)

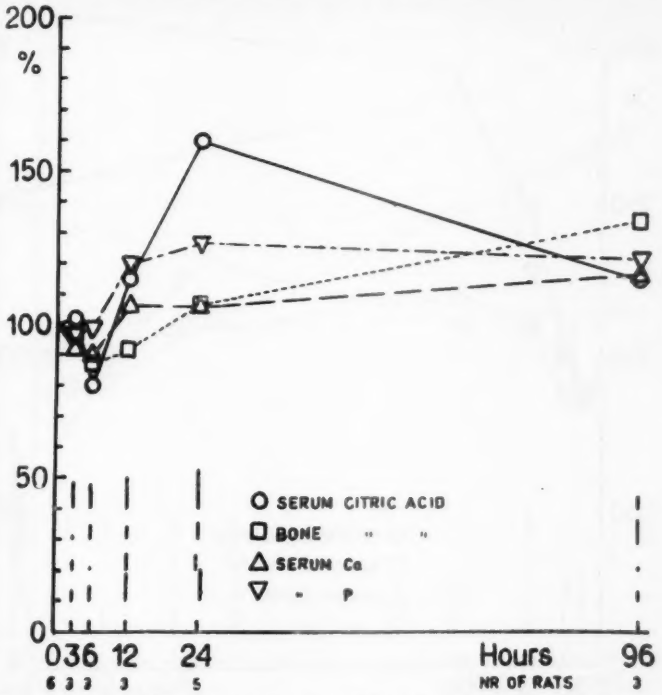

Fig. 1. Effect of a Physiological Dose (100 I.U.) of Vitamin D on Citric Acid and Mineral Levels in Rats Fed on a Diet with an Adequate Ca/P Ratio (0.8 per cent Ca and 0.5 per cent P in the Diet).
Columns below the curves indicate standard errors of the means. The columns for different samples stand on different levels as indicated in the Figure. Initial absolute values:

Serum citric acid	5.0 mg per 100 ml
Bone citric acid	30.4 micrograms per mg Ca
Serum Ca	9.1 mg per 100 ml
Serum P	7.3 mg per 100 ml

or Ca (Fig. 4) the rise in the serum citric acid was less rapid and did not differ appreciably from that occurring in the bones.

In all the experimental groups the rise in serum citric acid was preceded by a slight drop, observed during the first 6 to 12 hours following the vitamin D administration; in the group receiving 100,000 I.U. (Fig. 2) the changes occurred more rapidly and the drop was thus seen already after 3 hours.

The fluctuations in the serum citric acid occurring during the first few days following vitamin D seemed to be paralleled by

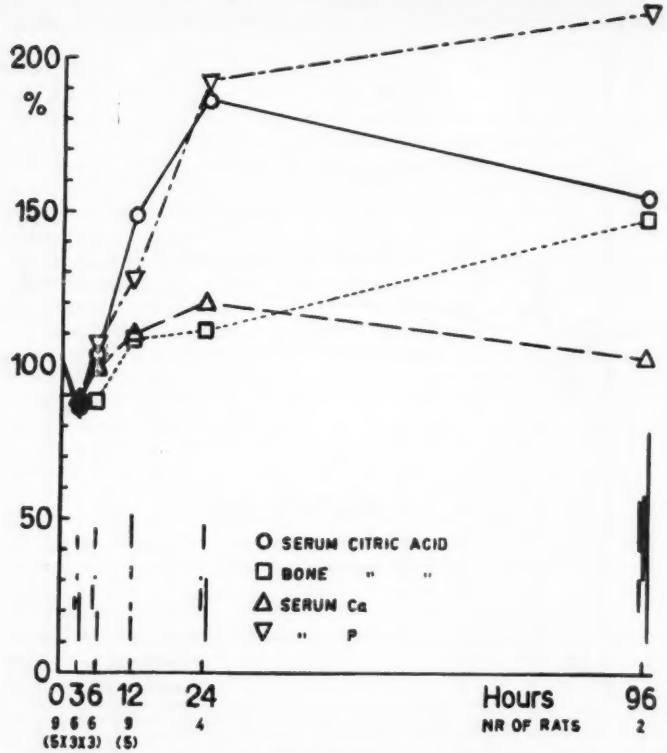

Fig. 2. Effect of an Excessive Dose (100,000 I.U.) of Vitamin D on Citric Acid and Mineral Levels in Rats Fed on a Diet with an Adequate Ca/P Ratio (0.8 per cent Ca and 0.5 per cent P in the Diet).
Small figures in brackets indicate number of observations for the bone citric acid when not estimated in all animals. The symbols are the same as in Figure 1. Initial absolute values:

Serum citric acid	5.2 mg per 100 ml
Bone citric acid	29.4 micrograms per mg Ca
Serum Ca	9.0 mg per 100 ml
Serum P	7.4 mg per 100 ml

changes in the levels of either serum Ca or serum P or both. Thus in the Ca deficient animals (Fig. 4) the Ca and citric acid levels followed each other, whereas the P level, which was normal from the beginning, remained unchanged. In the P deficient animals (Fig. 3) essentially the same picture was seen, except that the Ca and P had changed places. The P level thus behaved as the citric acid level, while the normal Ca level showed no marked

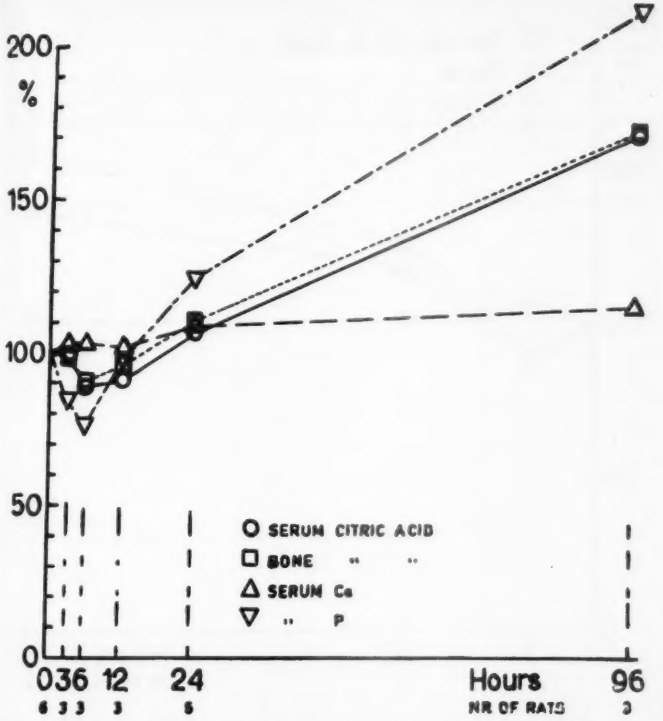

Fig. 3. Effect of a Physiological Dose (100 I.U.) of Vitamin D on Citric Acid and Mineral Levels in P Deficient Rats (2.0 per cent Ca and 0.5 per cent P in the Diet). Symbols are the same as in Figure 1. Initial absolute values:

Serum citric acid	5.3 mg per 100 ml
Bone citric acid	42.6 micrograms per mg Ca
Serum Ca	10.2 mg per 100 ml
Serum P	3.3 mg per 100 ml

change. When the diet was rich in both Ca and P (Figures 1 and 2), there seemed to be an effect on the levels of both serum minerals, although predominantly on the P level.

It should be noted that this parallelism between citric acid and Ca and/or P included the first period after vitamin D, when there was a drop in the serum citric acid. This inverse effect was studied separately in the experiment represented in Table 1. The animals in this experiment were kept on a diet rich in both Ca

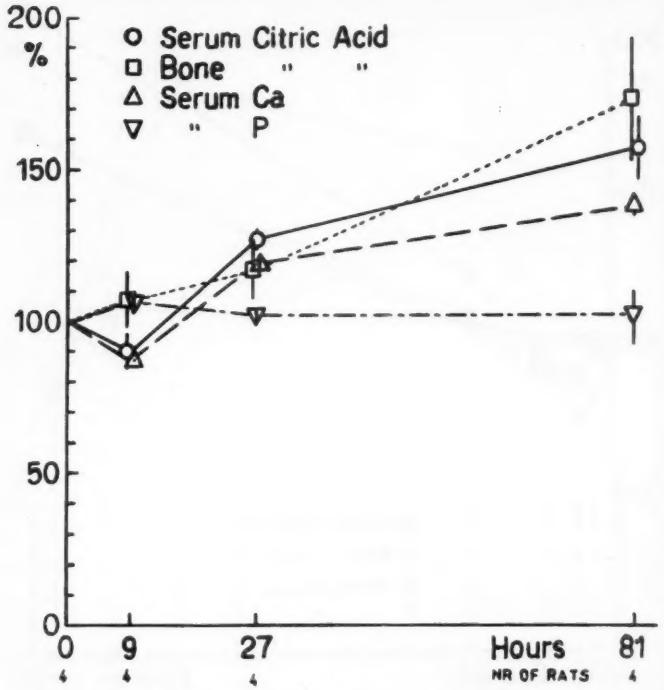

Fig. 4. Effect of a Physiological Dose (100 I.U.) of Vitamin D on Citric Acid and Mineral Levels in Ca Deficient Rats (0.04 per cent Ca and 0.5 per cent P in the Diet).
Each symbol with its column indicates a mean ± its standard error. Initial absolute values:

Serum citric acid	3.5 mg per 100 ml
Bone citric acid	20.7 micrograms per mg Ca
Serum Ca	5.7 mg per 100 ml
Serum P	7.6 mg per 100 ml

and P. There was a drop in both the serum citric acid and the serum P 6 hours after vitamin D administration. The Ca level, which was almost normal in this experiment, remained unaffected. There was no change in the bone citric acid.

Table 2 shows the action on the citric acid in serum and bones of vitamin D, after it had been given repeatedly during 3 weeks, the dose being 100 I.U. 3 times a week. The effect was found to be more pronounced in the Ca deficient animals than in the other groups. This particular behaviour of Ca deficient animals may be

Table 1.

Inverse Action of Vitamin D.

The animals which had been fed on a diet containing 0.8 per cent Ca and 0.5 cent P, were killed 6 hours after the administration of 100 I. U. of vitamin D.

	Vitamin D	Serum, mg per 100 ml			Bone Citric Acid	
		Ca	P	Citric Acid	μg	μg per mg Ca
	—	8.7	6.0	7.4	754	23.3
	—	9.3	5.9	6.3	656	25.1
	—	9.1	6.5	5.5	614	19.9
	—	9.4	7.1	5.1	760	22.2
	—	8.9	6.3	4.6	648	23.6
Average		9.1	6.4	5.8	686	22.8
	+	9.6	5.7	4.1	789	22.0
	+	8.8	4.5	4.0	628	22.1
	+	9.5	5.7	5.2	760	25.4
	+	9.7	5.6	3.6	679	22.6
	+	8.8	5.7	3.9	601	24.0
Average		9.3	5.4	4.2	691	23.2
Difference		0.2	— 1.0	— 1.6	5	0.4
± Standard Error		0.24	0.32	0.56		
P %		> 10	< 2.5	< 2.5	—	—
Difference %		2	— 16	— 28	1	2

referable to the very marked rise in the serum Ca caused by vitamin D. As will be shown below, the serum Ca level per se appears to have an influence on the citric acid of serum and bones. When this effect is accounted for, examination of Figures 1 to 4 and Table 2 gives the impression that the action of vitamin D on the citric acid contents of serum and bones tends to decline after reaching a maximum. It should be noted that the levels of serum Ca and P do not show a similar tendency.

The influence of the serum Ca and P on the citric acid of serum and bones was studied in experiments with varying Ca and P contents of the diet (Table 3). The animals were kept on the different diets for 3 weeks. Increasing the serum P by adding phosphate to the diet, from 2.3 to 6.9 mg per 100 ml, with a simultaneous increase of the Ca × P product from 23 to 57, was not accompanied by any detectable change in the serum citric acid. There was possibly a rise in the citric acid of the bones, but it was less than the simultaneous increase in bone Ca, resulting in a drop in the ratio citric acid/Ca. On the other hand, the serum Ca appeared to have a definite influence. The addition of Ca to the diet pro-

Table 2.

Effect of Prolonged Exposure to Vitamin D.

The vitamin was given in doses of 100 I. U. 3 times a week during 3 weeks.

	Dietary		Vit. D	Nr of rats	Body weight grams	Serum, mg per 100 ml			Bones		Incisors	
									Citric Acid		Citric Acid	
	Ca %	P %				Ca	P	Citric Acid	µg	µg / mg Ca	µg	µg / mg Ca
	0.8	0.5	—	5	89	8.8	5.6	4.3	572	23.6	96	3.0
	0.8	0.5	+	4	89	10.5	8.3	5.8	—	—	147	4.3
Difference					0	1.7	2.7	1.5	—	—	51	1.3
± Standard Error						—[1]	0.28	0.49	—	—	14.7	0.32
P %							< 0.1	< 2.5			< 2.5	< 0.5
Difference %					**0**	**19**	**48**	**35**	—	—	**53**	**43**
	2.0	1.5	—	5	93	8.2	6.9	4.8	699	27.1	143	4.7
	2.0	1.5	+	5	94	9.0	7.6	5.7	922	33.7	186	6.0
Difference					1	0.8	0.7	0.9	223	6.6	43	2.7
± Standard Error						0.50	0.31	0.39	57	1,38	16.6	0.55
P %						> 10	< 10	< 5	< 0.5	< 0.5	< 5	< 0.5
Difference %					**1**	**10**	**10**	**19**	**32**	**24**	**30**	**57**
	2.0	0.5	—	5	78	10.6	3.1	5.6	605	34.6	239	7.5
	2.0	0.5	+	5	74	12.6	5.0	6.6	717	34.5	341	10.2
Difference					— 4	2.0	1.9	1.0	112	— 0.1	102	2.7
± Standard Error						0.37	0.16	0.27	77	—	40	1.18
P %						< 0.1	< 0.1	< 1	> 10	—	< 5	< 10
Difference %					**— 5**	**19**	**61**	**18**	**17**	**0**	**43**	**36**
	0.04	0.5	—	4	68	4.7	—	3.5	211	20.7	66	3.1
	0.04	0.5	+	4	83	8.8	—	5.7	469	44.5	142	4.8
Difference					15	4.1	—	2.2	258	23.8	76	1.7
± Standard Error					1.9	0.11	—	0.32	28	1.70	4.2	0.2
P %					> 0.1	< 0.1	—	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Difference %					**22**	**87**	—	**63**	**123**	**115**	**115**	**55**

[1] Statistics omitted.

Difference					15	4.1	—	2.2	258	23.8	76	1.7
± Standard Error					1.9	0.11	—	0.32	28	1.70	4.2	0.2
P %					> 0.1	< 0.1	—	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Difference %					**22**	**87**	—	**63**	**123**	**115**	**115**	**55**

[1] Sparingly supplied.

Table 3.

Effect of Varying Ca and P Levels in the Diet.

The animals were kept on the different experimental diets for 3 weeks.

Dietary Ca %	Dietary P %	Vit. D	Nr of rats	Body weight grams	Serum, mg per 100 ml: Ca	Serum, mg per 100 ml: P	Serum, mg per 100 ml: Citric Acid	Bones: Citric Acid: µg	Bones: Citric Acid: µg / mg Ca	Incisors: Citric Acid: µg	Incisors: Citric Acid: µg / mg Ca
2.0	0.5	—	5	89	10.2	2.3	5.2	549	36.8	155	5.2
2.0	1.5	—	5	93	8.2	6.9	4.8	699	27.1	143	4.7
Difference				4	— 2.0	4.6	— 0.4	150	— 9.7	— 12	— 0.5
± Standard Error					0.45	0.30	0.34	78	1.7	17	0.4
P %					< 0.5	< 0.1	> 10	< 10	< 0.1	> 10	> 10
Difference %				4	**— 20**	**200**	— 8	**27**	**— 26**	— 8	**— 10**
0.04	0.5	—	5	55	—[1]	7.6	2.5	116	13.9	29	1.6
0.8	0.5	—	5	89	8.8	5.6	4.3	572	23.6	96	3.0
Difference				34	—	— 2.0	1.8	456	9.7	67	1.4
± Standard Error				8.3	—	0.31	0.30	71	1.6	5.8	0.18
P %				< 0.5	—	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Difference %				**62**	—	**— 26**	**72**	**394**	**70**	**231**	**88**

[1] Sufficient serum for Ca estimation not available. The value usually observed in animals on this diet is about 5 mg per 100 ml (compare Table 2 and Figure 4).

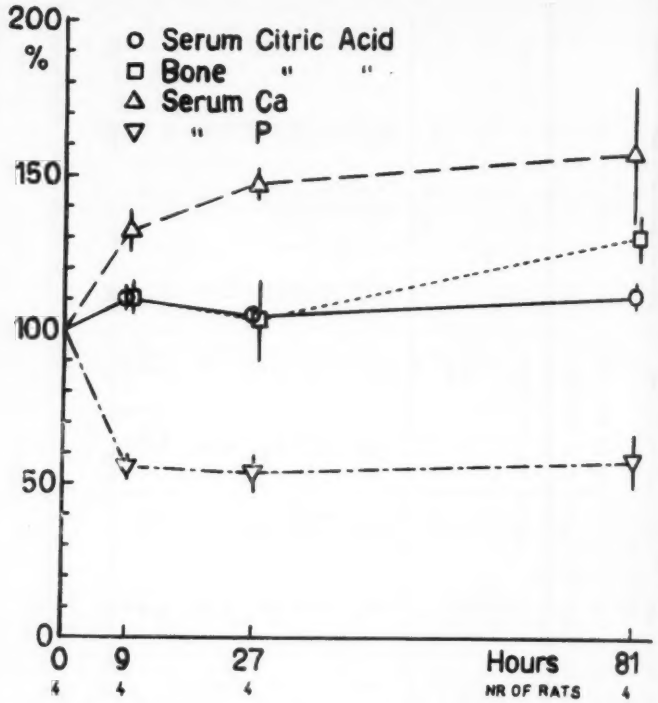

Fig. 5. Effect of Ca Addition to the Diet on Citric Acid and Mineral Levels in Ca Deficient Rats.
The initial values are the same as in Fig. 4. The animals were transferred to a diet containing 0.8 per cent Ca and 0.5 per cent P.

duced a rise in the serum citric acid from 2.5 to 4.3 mg per 100 ml, accompanied by an increase in the ratio citric acid/Ca in the bones.

Thus, while it did not seem probable that the action of vitamin D on the citric acid metabolism was the consequence of a rise in the serum P or in the serum product Ca × P, the possible rôle of the serum Ca as a causative factor did not appear unlikely. It was found, however, that the effect of the serum Ca developed too slowly to account for the rapid action of vitamin D on the serum citric acid. When Ca deficient rats were transferred to the diet containing 0.8 per cent Ca there was a rapid rise in the serum Ca, but during the first few days the citric acid in serum and bones remained unchanged (Fig. 5). These observations should be com-

Table 4.

Citric Acid Content of the Tip, Middle and Root Portions of the Incisors at Different Intervals after a Physiological Dose (100 I. U.) of Vitamin D.

The diet contained 0.8 per cent Ca and 0.5 per cent P. The 3 portions of the incisors were of equal length. Values for the tibia-fibula are given for comparison.

Days after vit. D	Nr of rats	Incisors								Bones	
		Tip		Middle		Root		Total			
		µg	µg / mg Ca	µg	µg / mg Ca	µg	µg / mg Ca	µg	µg / mg Ca	µg	µg / mg Ca
—	3	135	9.4	52	4.0	15	4.1	202	6.6	745	34.4
1	2	120	8.2	48	4.3	21	5.6	188	6.4	832	40.1
4	3	142	9.5	76	7.2	34	9.0	252	8.6	979	45.8

pared to the rapid parallel changes in the serum levels of Ca and citric acid after vitamin D administration to Ca deficient litter mates (Fig. 4).

In the incisors the citric acid/Ca ratio was 5 to 10 times lower than in the bones (Tables 2 to 4). This ratio also seems to be lower than those of the teeth of men, dogs and a number of other species examined earlier (LEICESTER 1949). So far, no explanation can be given to this remarkable phenomenon. In other respects the incisors appeared to behave as the bones: their citric acid content increased after vitamin D administration, the reaction being most rapid in the root portion (Table 4), and also varied with the Ca content of the diet.

Discussion.

In the present work it was demonstrated that vitamin D, when given in a physiological dose to vitamin D deficient animals, causes a rapid increase in the citric acid levels of blood serum and bones. These changes could not be explained as secondary to the action of vitamin D on the mineral metabolism.

It is obvious that the two effects of the vitamin, *i. e.* that on the bone minerals and that on the citric acid, are closely related to each other. According to FREEMAN and CHANG (l. c.) the simplest explanation of this relationship would be sought in the fact that the bone salt contains citric acid: when the solution of bone salt

is accelerated by vitamin D, the level of serum citric acid will increase along with the levels of the serum minerals. This explanation is not satisfactory. The citric acid/Ca ratio is about 10 times higher in the serum than in the bones. There is a similar difference in citric acid/P ratios. The simple mobilization of bone salt would therefore affect the serum citric acid much less than the levels of the minerals, which is in contrast to the changes observed after vitamin D administration. Further, this explanation does not take into account the increase in bone citric acid produced by the vitamin.

From the close parallelism between the serum levels of citric acid and the minerals in question it is, nevertheless, tempting to suggest, as did FREEMAN and CHANG, that they enter the blood stream together. The source of the Ca entering the blood stream as a consequence of vitamin D action is almost exclusively the bones, when the animals are kept on the Ca "free" diet (CARLSSON 1952). Even here, there was a close parallelism between the levels of serum citric acid and Ca. It therefore seems reasonable to suggest that the citric acid enters the blood stream in the bones together with the recently dissolved bone salt. In view of the differences in citric acid/Ca and citric acid/P ratios as well as the increase in bone citric acid discussed above, we are then forced to assume that citric acid is produced in the bones and that this process is accelerated by vitamin D. The physiological function of this production would be to accelerate the solution of bone salt. It is of interest in this connection to mention the work of DIXON and PERKINS (1952), who showed that the enzymatic apparatus necessary for the production of a local high concentration of citric acid exists in bone. As is well known citric acid increases the solubility of bone salt not only by decreasing pH but also through an effect of the citrate ion (KUYPER l. c.).

In the animals fed on a diet with a good Ca/P ratio the citric acid rose much more promptly in the serum than in the bones following vitamin D administration. This would seem contradictory to the view presented above, that the primary events occurred in the bones, but it should be noted that most of the bone citric acid is present in some kind of combination with the bone salt. Owing to this large fraction which is probably inert from a metabolic point of view, a high concentration of citric acid may well be present in small parts of the fluid phase of the bone tissue without any measurable influence on the total citric acid content

of the bone. The fairly slow rise in the citric acid content of the bones and incisors produced by vitamin D is probably secondary and represents an increase in the large inert fraction following the rise in the citric acid level of the fluid phase of the body.

The possible rôle of citric acid in the intestinal action of vitamin D has been discussed for many years (for references, see HARRISON and HARRISON l. c.). In a few experiments we failed to detect any marked effect of vitamin D on the intestinal citric acid, but certainly more work is required before the problem can be regarded as settled.

The action of vitamin D is generally said to set in fairly slowly, or even with a latency (cf. LINDQUIST 1952). In the present experiments we have observed for the first time an effect of the vitamin, which can be regarded as immediate, when the time necessary for absorption and distribution is accounted for. The effect comprises a drop in the levels of serum citric acid and Ca and/or P and may thus be described as an inverse action. It may be related to the tetany sometimes observed after the administration of vitamin D to rachitic infants and monkeys (GERSTENBERGER 1938). It might be taken as indicative of an early effect of the vitamin on the deposition of bone salt, but for reasons discussed above (citric acid/Ca and citric acid/P ratios) such an effect does not explain the fairly marked drop in the serum citric acid. One possibility would be that the first effect of the vitamin was to inhibit the production of citric acid in the bones, *i. e.* the opposite effect to that suggested to occur later. It may be that the vitamin has to be modified before it can exert its physiological effect and that the unchanged vitamin acts as an antimetabolite. There are certainly other possibilities to consider.

In conclusion, the data so far available seem to fit in with the hypothesis that the primary action of vitamin D is on the production of citric acid in the bones and that this effect explains the influence of the vitamin on the solution of bone salt.

Summary.

A new modification of the pentabromacetone method for the estimation of citric acid is described. One of the critical points in the method is avoided by substituting m-hydroxybenzoic acid for hydrogen peroxide.

The citric acid contents of serum and calcified tissues were followed at varying intervals after the administration of physiological or excessive doses of vitamin D to young rats, and were compared to the simultaneous changes in the levels of serum Ca and P. The influence of varying Ca and P contents of the diet was also studied.

Vitamin D caused a rapid increase in the citric acid contents of serum, bones, and incisors. The rise in the serum citric acid was in certain experiments more prompt than that observed in the bones. It was preceded by a drop. This inverse action of vitamin D seems to occur earlier than any other effect of the vitamin known hitherto.

The fluctuations in the serum citric acid following vitamin D administration were paralleled by changes in the levels of serum Ca and/or P depending partly on the Ca and P contents of the diet.

Marked changes in the serum P and in the serum product Ca $\times$ P caused by varying the P content of the diet did not affect the serum citric acid. Variation in the serum Ca induced parallel changes in the citric acid contents of serum and calcified tissues, but the effect developed too slowly to explain the corresponding action of vitamin D.

There is thus no evidence that the influence of vitamin D on the citric acid metabolism is secondary to the action on the mineral metabolism. It is suggested that vitamin D accelerates the citric acid production in the bones, which would explain the influence of the vitamin on the solution of bone salt.

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Metabolism of Bone Sodium in Rats Investigated with Na^{22}.

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It has long been known that sodium is present in bone principally in two fractions (HARRISON 1937[1]):

1) the fraction that can be accounted for on the basis of the extracellular water in bone.

2) the fraction that cannot be accounted for on this basis ("excess" sodium).

From radiosodium experiments it is known that a considerable part of this latter fraction does not enter into ionic exchange with the serum sodium (DAVIES *et al.* 1952 a; EDELMAN *et al.* 1952).

It is the purpose of the present investigation to determine

a) how large is this stable part of the excess bone sodium;

b) the rate of incorporation of sodium into the stable part.

For this purpose radioactive sodium (Na^{22}) has been administered to rats and the activity and sodium content of various bone samples and of the serum have been determined at varying intervals of time after the administration of the isotope. In order to gain information concerning the importance of bone growth for the accumulation of radiosodium in bone, one series of mature rats and one series of growing rats have been used for the investigation.

[1] *Note:* Though the technique of Harrison is open to criticism (see DAVIES *et al.* 1952 b), his main conclusion remains unopposed (see DAVIES *et al.* 1952 a).

Experiment.

The experiment was carried out in two series, each comprising twenty-one rats, both males and females of an inbred strain. Before and during the experiment the rats received a normal diet containing vitamins and minerals in adequate amounts.

The rats of *series 1* weighed on the average 218 g at the start of the experiment and increased insignificantly in weight during the 120 hour experimental time. The animals of this series were killed in groups of one to four at intervals of time after the administration of the isotope which ranged from 1—120 hours.

The rats of *series 2* weighed on the average 119 g and were six weeks old at the start of the experiment. They were therefore still in the period of relatively rapid growth. These animals were killed in groups of three at intervals of time after the administration of the radiosodium, ranging from one to sixty-four days.

Each rat in both the experimental series received a subcutaneous injection of about 90 μC Na^{22} in 1.5 ml of 0.9 % NaCl solution. The Na^{22} was supplied from Harwell and had been extracted from magnesium targets bombarded by the University of Birmingham's cyclotron. Before use it was kept for a month in a sealed vessel.

The rats were kept in separate cages after the injections and were killed by bleeding under ether anaesthesia at the respective intervals of time after the administration of the isotope. No signs of irradiation damage were observed in the animals during the experiment.

The blood was collected and after centrifugation, 1 ml of serum was mixed with 5 ml of 30 wt.% trichloroacetic acid (TCA). After centrifugation of this mixture, the clear supernatant liquid was secured.

Immediately after death of the animals, the femurs, the tibias, the humeri, and the incisor teeth were removed as soon as possible and dissected free from adhering soft tissues. The bone marrow was not removed from the bones, nor the pulp from the teeth. The femurs and the tibias were divided by a circular saw into ends and shafts. The levels of division were those indicated in fig. 1 in BAUER 1954 c.

From each animal the following samples were obtained:

Serum

A. Proximal femur ends and distal tibia ends ("non-growing ends").
B. Distal femur ends and proximal tibia ends ("growing ends").
C. Femur and tibia shafts.
D. One or both humeri.

The incisors were pooled to form the following samples in each group of rats:

E. Upper incisor teeth.
F. Distal halves of the lower incisor teeth.
G. Proximal halves of the lower incisor teeth.

In series 1 both humeri of each animal comprised sample D. In series 2 one humerus comprised sample D and the other humerus was used for radio-autography (see BAUER 1954 d).

Immediately after the bones and the teeth had been removed and divided to form the different samples, the samples were weighed. The samples were then dried in an electrically heated chamber at about 80° centigrade for at least 3 days. After drying, the samples were again weighed, introduced into calibrated test tubes and made up to 6 ml with 30 wt.% TCA. The tubes were vigorously shaken for 4 hours in a mechanical shaking device.

The TCA solution was then decanted into another set of stoppered test tubes. In order to find out whether the mineral content of the bone samples had been quantitatively dissolved in the TCA solution, the tissue residue from a great many tubes was collected, washed with distilled water and ashed in an electrically heated muffle at 800° centigrade for 3 hours. The weight of this ashed residue was calculated to be the equivalent of about 2 per cent of the total amount originally present in the tubes before treatment with the TCA.

The radioactivity of the TCA solutions was determined using the technique described by CARLSSON (1951) for the determination of the radioactivity of Ca^{45} solutions. However, a rate-meter was used instead of a scaler (see BAUER 1954 a).

The radioactivity of Na^{22} is partly γ rays. Therefore no indefinitely thick layer of the Na^{22}-solutions could conveniently be used for the determination of the radioactivity of the solutions. The radioactivity was measured on 3 ml of the TCA-solution in standard cups. It was found that a pipetting error of ± 0.1 ml would give a measuring error of about ± 1 %. The error introduced by any reasonable pipetting error was thus small compared to the statistical error involved in radioactive assay.

The standard deviation of the activity measurements was always kept less than 5 per cent of the counting rates encountered. A standard solution containing the dose in 1,000/3 ml was used for reference. This standard gave a counting rate of about 30 c. p. s. The background was always less than 0.8 c. p. s. The background determinations were always performed with a set of samples a standard distance from the counting device (about 1 meter). No radioactive source other than the sample actually counted was permitted ever to get within this distance. When necessary the samples were diluted with 30 wt.% TCA prior to the activity measurements.

If not stated otherwise, in this paper the amounts of activity recovered from the respective samples have been expressed as per cent of the dose administered.

The calcium and sodium determinations were made according to the flame photometry method described by BAUER, 1954 b. The sodium content of the serum was determined by flame photometer on dilute aliquots of the serum TCA mixture used for the radioactivity measurements.

Results.

Series 1. (Mature rats killed 1—120 hours after a subcutaneous injection of Na^{22}.)

In table 1 are recorded the average wet and dry weights, Ca and Na content of all the samples of series 1.

Table 1.

Average Mineral and Water Content of Different Samples of Bones and Incisor Teeth in Mature Rats.

	A	B	C	D	E	F	G
Fresh weight mg	510	900	679	555	368	210	170
Dry » »	320	469	462	326	254	175	95
Water content mg	190	431	217	229	114	35	75
» as % of fresh weight	37.2	47.9	32.0	41.2	31.0	16.7	44.1
Calcium content mg	56.2	71.3	130	73.5	73.7	49.4	24.4
» as % of dry weight	17.5	15.2	28.2	22.6	29.0	28.2	25.6
Sodium content mg	1.70	2.64	3.27	2.16	2.35	1.40	0.95
» as % of dry weight	0.53	0.56	0.71	0.66	0.93	0.80	1.00
Water phase sodium mg ..	0.65	1.46	0.74	0.78	0.39	0.13	0.27
Ca/Na_{excess} weight ratio ..	53.6	60.4	51.4	53.3	37.6	38.9	35.6

In the above table are listed the average values of the following samples from twenty-one animals:

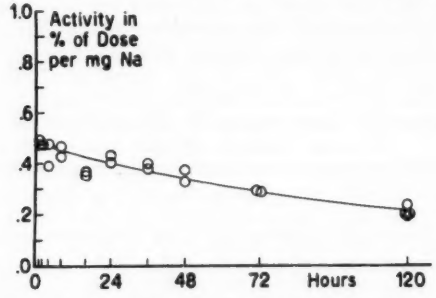
A. Proximal femur ends and distal tibia ends (*non-growing ends*) pooled from one animal.
B. Distal femur ends and proximal tibia ends (*growing ends*) pooled from one animal.
C. Femur and tibia shafts pooled from one animal.
D. Humeri pooled from one animal.
E. Upper incisor teeth pooled from two animals.
F. Distal halves of the lower incisor teeth pooled from two animals.
G. Proximal halves of the lower incisor teeth pooled from two animals.

In graph 1 is recorded the specific activity of the serum sodium at the ends of the different intervals of time after the administration of the isotope. It is seen that there is a continuous but slow fall in this value.

In graph 2 the specific activity of the sodium of the different bone samples is plotted as per cent of that of the serum sodium. It is seen that after a fast rise, these "exchange values" stay practically unchanged from 16 hours after the administration of the radiosodium. Even at the longest interval of time (120 hours) no change in these values is observed.

Graph 1.

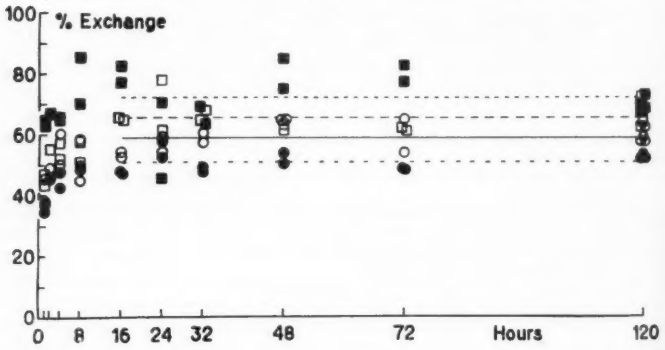
Specific Activity of the Serum Sodium at Varying Intervals of Time After the Administration of Na^{22} to Mature Rats.



Note: The value of each animal has been indicated in the graph.

Graph 2.

Radiosodium Exchange[1] *of Different Bone Samples*[2] *at Varying Intervals of Time After the Administration of Na^{22} to Mature Rats.*



Explanation of symbols.

■ - - - - - - - Growing ends

□ — — — — Non-growing ends

○ ——— Humeri

● - - - - Shafts of the tibias and the femurs

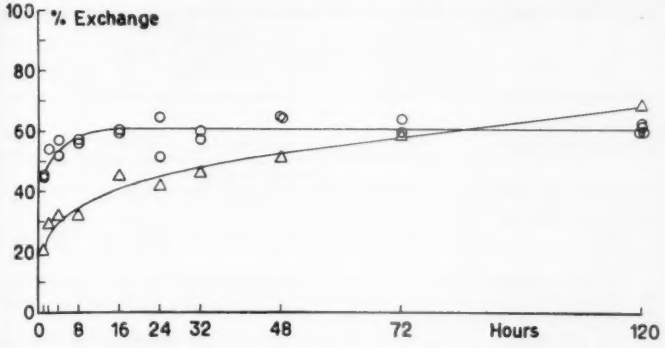
[1] Radiosodium exchange means the bone sodium specific activity (per cent of dose per mg sodium) expressed as per cent of the specific activity of the serum sodium (the serum values appear in graph 1). The exchange values of all the different bone samples are indicated in the above graph.

[2] The composition of the different bone samples are explained in detail in the text (page 335) and in the legend of table 1.

Note: The horisontal lines (one for each of the four different bone samples) represent the numerical averages of the 16—120 hour exchange values.

Graph 3.

Radiosodium Exchange[1] of the Skeleton[2] and the Incisor Teeth[3] at Varying Intervals of Time After the Administration of Na^{22} to Mature Rats.



Explanation of symbols.

○ Skeleton
△ Incisor teeth

[1] Consult the legend of graph 2 for an explanation of the term radiosodium exchange.

[2] The specific activities of the skeleton have been calculated for each animal from the pooled activities and sodium content of the femurs, the tibias, and the humeri.

[3] The specific activities of the incisor teeth have been calculated from the pooled activities and the sodium content of the incisors of all animals in each group.

In graph 3 it is seen that the sodium exchange values of the incisor teeth do not stay constant during the 16—120 hour period. After an initial rapid rise to a 24 hour value of 40—50 %, there is a slow but continuous rise to a 120 hour value of 60—70 %.

The numerical averages of these exchange values of the different samples (as calculated from all groups between the 16 hour interval and the 120 hour interval inclusive) have been introduced in table 2. It is seen from this table that the "steady state" exchange value is highest for the samples B (growing bone ends) and lowest for the samples C (the shafts of the tibias and the femurs).

If, however, the amount of sodium ascribable to the amount of water in the samples is supposed to have a specific activity the same as the serum sodium, it is seen in table 2 that the specific activity ratio of the excess bone sodium/serum sodium is almost identical for the different samples of bone and teeth, regardless of location.

Table 2.

Exchangeable Sodium[1] *of the Incisor Teeth and the Different Samples of Bone*[2] *in Mature Rats.*

	Non-growing ends	Growing ends	Shafts of the tibias & femurs	Humeri	Inc. teeth
Total sodium steady state exchange[3]	64.6 %	72.0 %	50.8 %	58.7 %	52.2 %
		average 60.8 %			
Na_{H_2O} as % of $Na_{tot.}$ ..	38.2 %	55.3 %	22.6 %	36.1 %	16.8 %
Na_{excess} steady state exchange	42.7 %	37.4 %	36.5 %	35.4 %	42.6 %

[1] Sodium exchange means the bone sodium specific activity (per cent of dose per mg sodium) expressed as per cent of the specific activity of the serum sodium (the serum values appear in graph 1).

[2] The composition of the different bone samples are explained in detail in the text (page 335) and in the legend of table 1.

[3] "Steady state" exchange is calculated as the mean exchange of the 16 hour group—120 hour group inclusive (see graph 2). The figures for the incisors are calculated in the same way though no plateau exchange value seems to be reached for the sodium of the incisor teeth (see graph 3).

From table 1 it can further be seen that this excess sodium can be calculated to represent a Na/Ca weight ratio of 1/35—1/60. This ratio is in agreement with the statement of HARRISON, l. c., that the molar ratio of the non-water sodium to calcium in the calcified tissues is about 1/30, *i. e.* a Na/Ca weight ratio of about 1/50.

Series 2. (Growing rats killed 1—64 days after a subcutaneous injection of Na^{22}.)

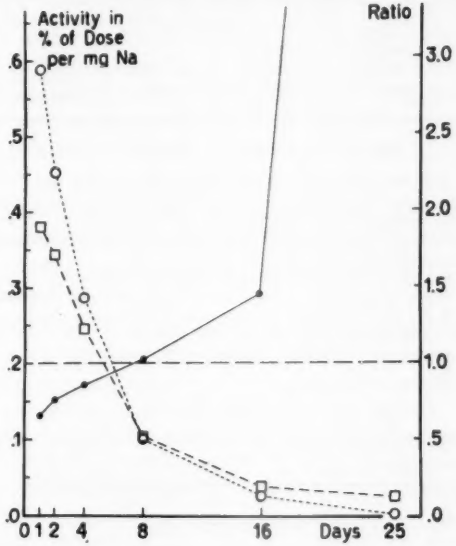
In graph 4 are recorded the specific activities of the bone and serum sodium at intervals of time after the administration of the isotope ranging from one to twenty-five days. It is seen from the graph that the specific activity of the serum sodium falls at a more rapid rate and to lower values than does the specific activity of the skeletal sodium.

Sixty-four days after the administration of the isotope, the serum activity could not be distinguished from the background counting rate. At this time the activities of the bone samples were still measurable (see table 3) though these solutions contained less sodium than did the serum solutions.

The specific activity ratio bone/serum sodium thus rose through all groups of this series to a final value far above unity.

Graph 4.

Specific Activity of the Skeletal[1] and the Serum Sodium[2] at Varying Intervals of Time After the Administration of Na^{22} to Growing Rats.



Explanation of symbols.

□ - - - □ Specific activity of the skeletal sodium

○ - - - - ○ Specific activity of the serum sodium

● ——— ● Specific activity ratio of the skeletal/serum sodium

[1] Calculated from the pooled activities and sodium content of the femurs, the tibias, and one humerus of the three rats of each group.

[2] Average of the three rats of each group.

Note: The scale to the right of the graph shows the numerical value of the specific activity ratio of the skeletal/serum sodium.

In table 4 are recorded the sodium specific activity ratios of the different bone samples to the serum at the varying intervals of time after the administration of the isotope. It is seen that the values of these activity ratios as recorded at day one, are similar to those recorded as the "steady state exchange values" of the bone sodium appearing in table 2 (mature rats).

From table 4 it can further be computed that even after the serum specific activity has become negligible, there is a more marked fall in the specific activities of the ends than in those of the shafts of the long bones. From table 3 it is seen that sixty-

Table 3.

Specific Activities[1] of the Sodium of Different Samples of Bone[2] Sixty-Four Days After the Administration of Na^{22} to Growing Rats.

Average weight of the animals	Dry weight of the humerus	Sodium specific activities of the			
		non-growing ends	growing ends	shafts of tibias & femurs	humerus (whole bone)
191 g	152 mg	0.0173	0.0129	0.0197	0.0181

[1] Specific activity means per cent of dose per mg Na.

[2] The above values have been calculated from the total activities and sodium content of the different bone samples of the three rats killed sixty-four days after the administration of the Na^{22}. See table 1 for an explanation of the composition of the different bone samples.

Note: Of the twelve different bone samples of this group of rats, no sample had a lower counting rate than 0.91 c. p. s. The three shaft samples had a counting rate of 2.19, 1.37 and 1.42 c. p. s. resp. The three serum samples had a counting rate of 0.71, 0.66 and 0.66 c. p. s. resp.

The background during these measurements was 0.666 c. p. s. with a S. D. of 0.0027 c. p. s.

Table 4.

Sodium Specific Activity Ratio of the Bone Samples to the Serum at Varying Intervals of Time After the Administration of Na^{22} to Growing Rats.

Days after the administration of the Na^{22}	Average weight of the animals at death	Dry wt. of the humerus	Non-growing ends	Growing ends	Shafts of the tibias and femurs	Humerus
1	119 g	86.6 mg	0.652	0.740	0.550	0.629
2	119 »	85.3 »	0.742	0.854	0.689	0.685
4	112 »	84.0 »	0.888	0.888	0.770	0.912
8	125 »	94.7 »	0.941	1.13	0.971	1.06
16	133 »	99.3 »	1.33	1.58	1.48	1.25
25	136 »	104 »	10.9	9.10	15.3	10.6

The composition of the different bone samples are explained in detail in the text (page 335) and in the legend of table 1.

four days after the administration of the isotope, the specific activity of the bone sodium is about 1.5 times higher in the shafts than in the growing ends of the long bones.

Discussion.

HARRISON showed that the excess sodium of bone (the sodium which cannot be accounted for as extracellular water sodium) cannot be extracted with water.

The present investigation shows that this fraction of the bone sodium in mature rats is only partly reached by the radiosodium of the serum. From table 2 and graph 2 it is seen that about one third of this sodium fraction rapidly reaches an activity equilibrium with the serum sodium. No further exchange occurs during the period of time from 16 hours to 120 hours after the administration of the radiosodium. The remaining two thirds of this excess sodium seem therefore to represent a stable, non-exchangeable sodium fraction. These figures are in accord with *inter al.* DAVIES *et al.* (1952 a), EDELMAN *et al.* (1952).

From the work of EDELMAN *et al.* who made a similar experiment with adult dogs, it can be seen that after the initial twenty-four hours there is no extra exchange even during a total period of thirty days.

In the experiment of EDELMAN *et al.* the serum specific activity dropped but 12 per cent during this thirty day period. In the present experimental series, the serum specific activity dropped more than 50 per cent during a five day period. The difference between these data is explained by the fact that the dogs of EDELMAN were given a diet deficient in sodium while the animals of the present investigation received a normal diet. Similar data as to the extent of the exchangeable fraction of the bone sodium were thus obtained, whether or not the animals were kept on sodium deficient diets.

The present investigation has shown that a considerable part of the skeletal non-water sodium is stable and non-exchangeable. In the *young* animals the ratio of the specific activity of the skeletal sodium to that of the serum sodium showed a marked rise during the experiment (see table 4 and graph 4). Thus while at day one this ratio was equal to that found to be the steady state exchange in the mature animals, at day twenty-five the specific activity of the skeletal sodium was about ten times higher than that of the serum sodium. Essentially the same findings were recorded for the incisor teeth (see table 5).

Sixty-four days after the administration of the radiosodium, practically no labelled sodium remained in the serum while the amount of radiosodium at least in the shafts of the long bones remained at about the twenty-five day level.

During the sixty-four day experimental time the rats increased considerably in size (the dry weight of the humerus nearly doubled from 85 mg to 152 mg). The formation of new bone tissue implies

Table 5.

Activity Values, Sodium and Calcium Content of the Incisor Teeth at Varying Intervals of Time After the Administration of Radiosodium to Growing Rats.

Days after the administration of Na^{22}	Activity in per cent of dose	Mg Na	Mg Ca	Ca/Na weight ratio
1	1.34	3.87	131	33.9
2	1.25	3.75	136	36.3
4	0.936	3.66	143	39.0
8	0.516	3.89	154	39.6
16	0.266	4.74	174	36.7
25	0.177	4.86	179	36.9

In this table are recorded the values derived from the pooled teeth of each group of three animals of series 2.

that a certain amount of labelled sodium was incorporated in the stable part of the bone sodium during the experimental time. This explains why the sodium specific activity ratio bone/serum reached values above unity.

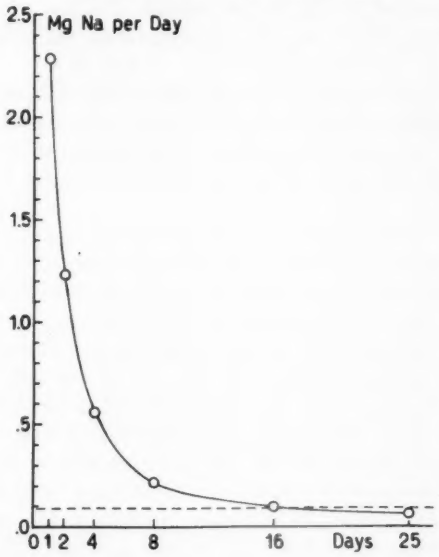
The continuous rise in the exchange values recorded for the teeth of the mature rats (see graph 3) can probably be explained along similar lines: The incisor teeth of rats never stop growing from the bases and are continuously worn off at the tips. The life cycle of the incisors of mature rats is about 40—50 days. About 1/10 of the entire tooth must therefore be replaced in 120 hours (the series 1 experimental time). This means that 1/10 of the stable (non-labelled) sodium of the teeth was replaced by labelled sodium during this period of time.

From a knowledge of the mean serum specific activity from the time of the administration of the radiosodium to the time of death of the respective animals, the rate of incorporation of sodium into the stable (non-exchangeable) fraction of the bone or teeth sodium may be computed:

Let U mean the amount of sodium in mg which is incorporated per day in the stable fraction in a certain sample of bone or tooth, C mean the absolute activity of the sample, S mean the average specific activity of the serum sodium during the interval of T days after the administration of the radiosodium. If an irreversible incorporation of sodium had occurred at a constant rate, the following formula should give a constant value $U = \frac{C}{T \times S}$ (see CARLSSON 1952 and BAUER 1954 c).

Graph 5.

Rate of Formation[1] of the Stable Part of the Sodium of the Incisor Teeth[2] as Calculated[3] at Varying Intervals of Time After the Administration of Radiosodium to Growing Rats.



Explanation of symbols.

○———○ Formation rate calculated from the activity values

– – – – – – Formation rate calculated from the rate of bone salt deposition in the teeth (see the text).

[1] The formation rate has been expressed in terms of mg Na per day.
[2] Calculated on the pooled teeth of three animals in each group.
[3] The formula used for this calculation is explained in the text.

In graph 5 the values obtained for U, at the different intervals of time investigated, are recorded for the pooled incisor teeth of the three rats of each group of series 2. It is seen from this graph that after a rapid decrease in this value, there is a plateau during the interval of 16—25 days after the administration of the radiosodium. This plateau has a value of 0.078 mg Na per day if calculated as the mean value of the 16 and 25 day values.

The stable fraction makes up about one half of the total sodium of the incisors (table 2). The weight ratio of the total sodium/calcium of the incisor teeth of the growing animals of

series 2 was on the average 1/37 (table 5). The four incisor teeth of a two month old rat deposit about 0.09 mg Ca per hour (CARLSSON 1952 and BAUER 1954 c). From these figures it can be computed that an amount of 0.087 mg sodium per day was incorporated in the stable sodium fraction of the pooled incisors of the three rats. A horizontal line has been indicated at this level in graph 5.

The agreement of these different sets of data makes it probable that the stable sodium is formed along with formation of the tooth tissue and that this sodium is not subject to removal until the corresponding part of the tooth is reached by the attrition process.

From the data plotted in graphs 4 and 5 it appears that the deposition rate of sodium in the calcified tissues cannot be calculated from the above formula until the labile fraction has reached activity values which are low compared to the amount of activity incorporated in the stable (non-exchangeable) part of the bone sodium. In figure 5 the part of the total value that lies below the horizontal line (the level of the plateau) indicates the proportion of the activity recovered that is due to the deposition of stable tooth sodium. The fraction above the horizontal line is proportional to the amount of activity that is ascribable to the effect of atomic exchange (including the water phase sodium).

It can be seen from the curve that the ratio of the exchange fraction (of the activity recovered) to that of the stable fraction (of the activity recovered) diminishes to values near zero when the plateau is reached. In this Na^{22} experiment this value was not reached until 16—25 days following the administration of the isotope. When Ca^{45} is used, this interval of time after the administration of the isotope when the above U-formula permits a reliable calculation of the deposition rate of bone salt in the teeth, is only 8—16 hours (see CARLSSON 1952). The explanation for this difference is that (in a radiocalcium experiment relative to a radiosodium experiment) the following factors tend to decrease this interval:

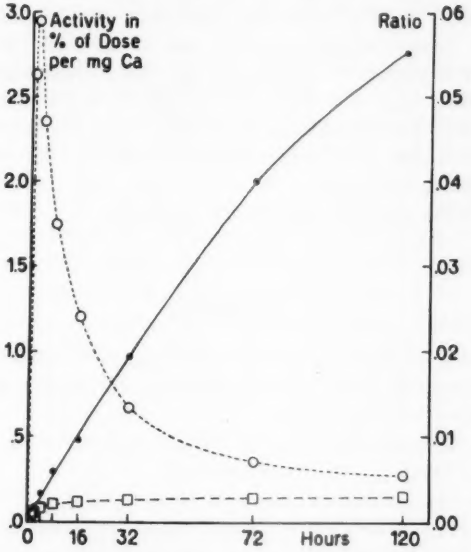
a) A more rapid fall in serum specific activity;

b) The ratio of the exchangeable fraction (including the water phase fraction) to the non-exchangeable fraction is lower.

Thus, when Ca^{45} is administered to mature rats, no steady state level is reached for the specific activity ratio of the skeleton/

Graph 6.

Specific Activity of the Tibia[1] *and the Serum Calcium*[2] *at Varying Intervals of Time After the Administration of* Ca^{45} *to Mature Rats.*
(The values appearing in this graph have been derived from an earlier experiment (see BAUER *1954 c)).*



Explanation of symbols.

□ — — — □ Specific activity of the calcium of the tibia
○ - - - - - ○ Specific activity of the calcium of the serum
● ——— ● Specific activity ratio of the skeletal/serum calcium

[1] Each value is calculated from the pooled activities and ash content of one tibia shaft in each of two animals.
[2] Average of the two animals of each group.
Note: The scale to the right of the graph shows the numerical value of the specific activity ratio of the tibia/serum calcium.

serum Ca; The ratio of the stable/labile fractions of the total activity recovered from the skeleton rapidly rises. In graph 6 is recorded the calcium specific activity ratio of the tibia shafts/serum of a previous experimental group (BAUER, 1954 c). There is a rapid rise in this ratio. The same fact was reflected by the appearance of the curve drawn through the "U-values" recorded for the incisors of the same animals. After an initial rapid fall,

a steady level was reached already at 8—16 hours after the administration of the radiocalcium.

A further evidence for the opinion that the radiosodium of the stable fraction (deposited with the formation of bone tissue) is not removed until this bone tissue is reached by a resorption process is furnished by the radio-autographs prepared from the humeri of the growing rats of series 2. As this part of the investigation has been published in detail elsewhere (BAUER 1954 d), only the 4 and 16 day autograms are shown here (fig. 1). It is seen how the image of the shaft and the slowly growing metaphyseal end of the humerus is clearly visible even in the 16 day autogram while at this time the image of the actively growing bone end has practically vanished.

Summary.

Sodium is present in bone in principally two fractions:

1) The fraction that can be accounted for on the basis of the extracellular water in bone.

2) The fraction that cannot be accounted for on this basis (excess sodium).

It was the purpose of the present investigation to determine

a) to which extent the excess sodium is available for rapid atomic exchange with the serum (extracellular water) sodium;

b) the rate of renewal of the stable (non-exchangeable) part of the excess sodium of bone.

Two series of rats received subcutaneous injections of Na^{22}. Series 1 comprised mature rats which were killed at short intervals of time (1—120 hours) after the administration of the isotope. Series 2 comprised young, growing rats which were killed at long intervals of time (1—64 days) after the administration of the isotope.

In both series of rats various samples of the long bones and the incisor teeth were analysed for their activity and sodium content. Serum samples were also analysed.

On the assumption that the extracellular water sodium of bone is freely exchangeable with the serum sodium, the following conclusions were reached:

1) 30—40 per cent of the "excess" sodium of bone is exchangeable with the extracellular water sodium.

2) No further exchange occurs after the initial 16 hours following the radiosodium administration to mature rats.

3) The non-exchangeable bone sodium is deposited with the formation of the bone or tooth tissue.

4) The non-exchangeable bone sodium stays unremoved until the corresponding part of the bone or tooth tissue is reached by resorption or attrition.

5) From a knowledge of the mean serum sodium specific activity from the time of the administration of the radiosodium to the time of death of the animals, the rate of incorporation of sodium in the stable fraction of the bone or teeth sodium may be computed.

6) A comparison with the same computation in a radiocalcium experiment reveals some fundamental rules governing the relative importance of irreversible and reversible processes for the uptake of radioactive indicators in the calcified tissues.

A knowledge of these mechanisms permits a computation of the rate of formation of tissues from tracer data.

A grant from the P. Håkansson Foundation, Eslöv, made possible the purchase of the Na^{22}.

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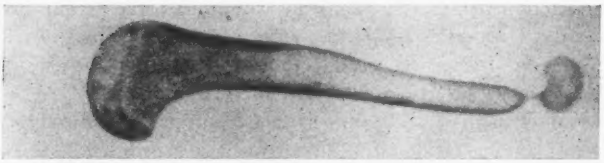
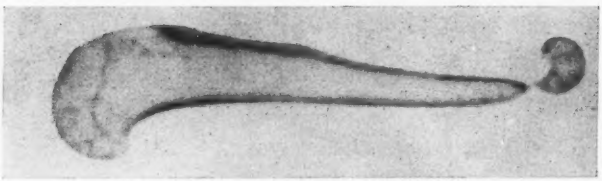
Fig. 1.

Humerus Radio-Autograms From Growing Rats Killed Four Resp. Sixteen Days Following the Administration of Na^{22}.



A. Killed 4 days after isotope injection.



B. Killed 16 days after isotope injection.

Note: The autograms of this plate have been magnified 4 times their original size.

(Courtesy, Acta Orthopedica Scandinavica.)

From the Orthopaedic Clinic, Malmö, and the Pharmacologic Institute of the University of Lund, Sweden.

A Rapid Method for the Simultaneous Determination of Calcium and Sodium in Bone.

By

GÖRAN C. H. BAUER.

(Received 22 January, 1954.)

In bone the calcium content is about forty times the sodium content if these values are expressed in weight per cent. When determinations of the sodium content of bone solutions are performed by flame photometry, the calcium light will interfere with the sodium light even when this is measured with an appropriate filter.

DAVIES *et al.* (1952) published a method based on flame photometry, to determine the sodium content of bone dissolved in nitric acid. The principle of their method is: "Na^{24} is added to the bone solution, interfering substances are removed by precipitation and sublimation and a correction made, by measurement of the residual radioactivity, for the loss of sodium."

This method is better than earlier methods which do not seem to have taken into account sources of error involved in the determination of sodium in bone (DAVIES *et al.*, l. c.).

However, for obvious reasons it is cumbersome to use the short-lived Na^{24}. Even if Na^{22} were used instead, the method might prove difficult if the bone samples that are to be analysed, contained radioactivity.

In our laboratory we investigate the uptake of radioactive calcium and sodium in the skeleton of rats. The radioactivity is measured in trichloroacetic acid (TCA) solutions of the bone samples. The following method makes it possible to determine the

sodium content of these solutions by flame photometry. Dilution of the bone solutions is the only extra step involved.

Method.

The principle is as follows:

1) The concentration of Ca in the unknown is determined by flame photometry.

2) The concentration of Na in the unknown is determined by flame photometry, using as a standard a synthetic bone solution. The emittance of Ca light of this standard is that determined in step 1).

Apparatus.

A Beckman DU spectrophotometer is used with the flame photometer attachment model 10300. Ca is read at 4,227 Å and Na is read 5,890 Å. The blue sensitive phototube is generally used. A 10,000 megohm phototube load resistance is employed and the selector switch is set at 0.1. The sensitivity knob is usually operated at mid-point. The flame burns with a mixture of natural gas (butan) and oxygen.

Within convenient concentration ranges of Ca and Na there does not exist any "plateau" for either air or oxygen pressure. Variations in these pressures therefore cause fluctuations in the readings obtained. We use an additional pressure regulator to keep the oxygen pressure at a constant value.

All investigations mentioned in this paper were made with an oxygen pressure of 30 inches of water and an air pressure of 29 lbs/sq. inch.

The reproductibility of the flame photometer readings is good. A deviation of more than 2—3 per cent from a standard value during an experimental series, makes one suspect a fault in the apparatus. The only frequent fault is clogging of the capillary.

Analyses of synthetic mineral solutions.

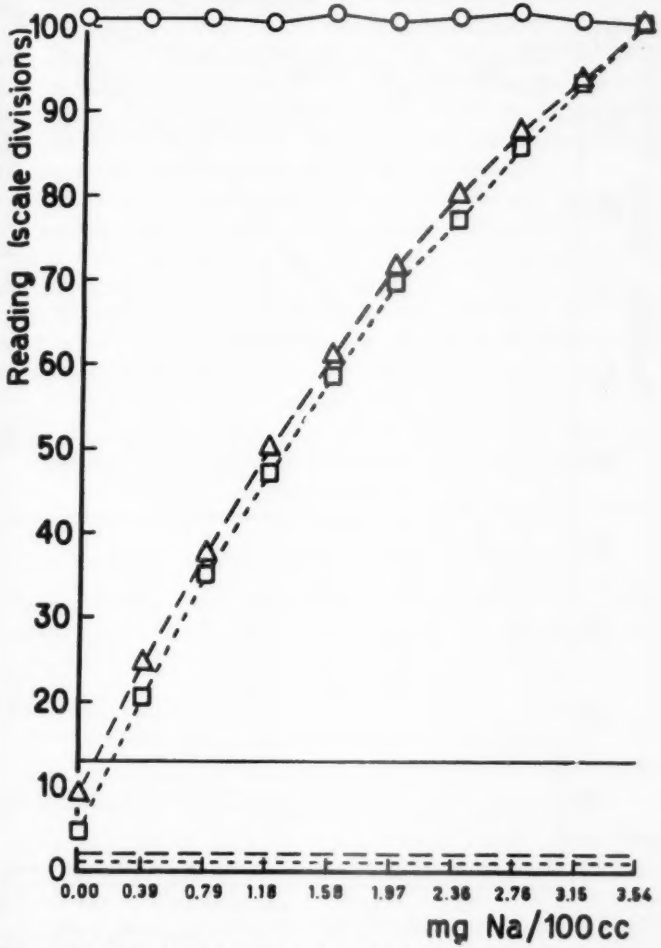
Synthetic mineral solutions have been prepared with $Ca_3(PO_4)_2$ dissolved in a TCA solution and NaCl dissolved in aq. dest. Unless stated otherwise, the solutions contain TCA in a concentration that does not exceed 1 per cent.

The examinations warrant the following conclusions. (For a further discussion of the interference phenomena see LUNDGREN 1953.)

1) Na does not appreciably interfere with the Ca readings when the weight ratio Ca/Na exceeds 11 : 1 (see figure 1).

Figure 1.

Flame Photometer Readings Obtained at Different Wavelengths with Solutions of a Constant Calcium and a Varying Sodium Concentration.



The concentration of Ca in all solutions tested was 40 mg/100 cc. Between each reading the instrument was calibrated with the 3.54 mg/100 cc Na solution.

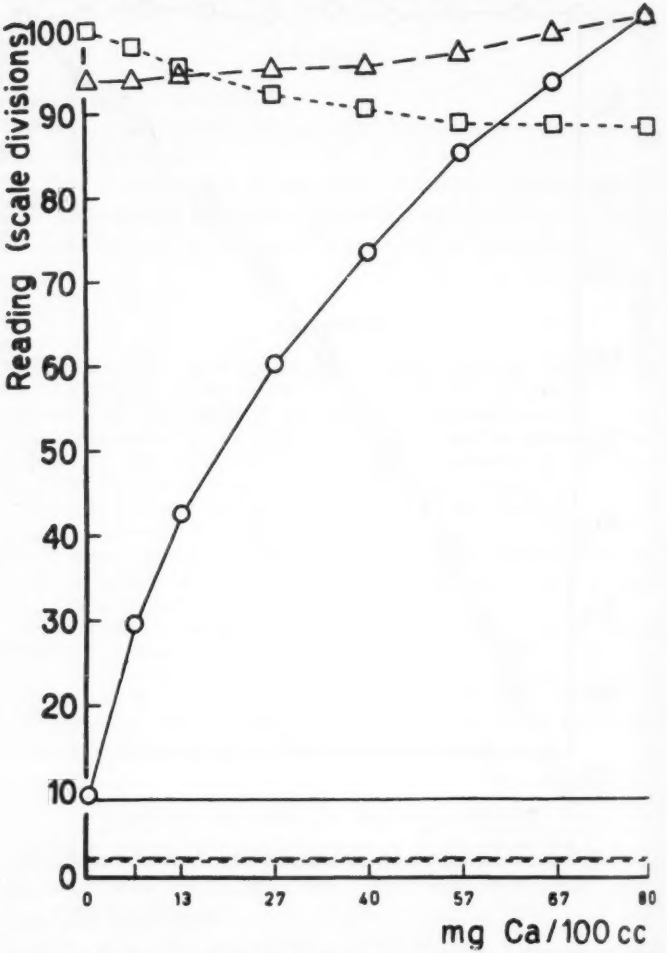
Explanation of symbols:

	Wavelength Å	Slit width mm	Phototube
○——○	4,227	0.2	blue sensitive
□ - - - □	5,893	0.05	» »
△ — — △	5,893	0.4	red »

The horizontal lines represent the background at the different settings.

Figure 2.

Flame Photometer Readings Obtained at Different Wavelengths with Solutions of a Constant Sodium and a Varying Calcium Concentration.



The concentration of Na in all solutions tested was 1.18 mg/100 cc. Between each reading the instrument was calibrated at the different wavelengths with the solution that gave the highest reading.

Explanation of symbols:

	Wavelength Å	Slit width mm	Phototube
○——○	4,227	0.2	blue sensitive
□ - - - □	5,893	0.1	» »
△ — — △	5,893	0.5	red »

The horizontal lines represent the background at the different settings.

2) The Ca readings are linearly related within a wide range of Ca concentrations (see figure 2).

3) Ca interferes with the Na readings (see figure 2). A 100 per cent rise in Ca concentration causes a depression of about 4 per cent in the Na readings when Na is read with the blue sensitive phototube. This interference is even less when Na is read with the red sensitive phototube. However, the interference during the latter condition causes a rise in the Na readings.

4) When the Ca concentration is kept constant, the Na readings are linearly related within a wide range of Na concentrations. This is true whether Na is measured with the blue sensitive or the red sensitive phototube (see figure 1).

5) Wide fluctuations of the TCA concentrations of the solutions cause but small fluctuations in the Ca or Na readings obtained (see table 1).

Table 1.

Flame Photometer Readings Obtained at Different Wavelengths with Solutions of a Varying Trichloroacetic Acid Concentration.

Reading obtained at

TCA conc.	4,227 0.1 Blue	5,893 0.05 Blue	5,893 0.5 Red	Wavelength Å Slit width mm sensitive phototube
0.9 %	100	100	100	
2.9 %	102	103	100	
10.9 %	107	105	106	
20.9 %	107	104	107	
Background	7	5	5	

The concentration of Ca in all solutions tested was 40 mg/100 cc.
» » » Na » » » » » 1.18 mg/100 cc.
At the resp. wavelengths the instrument was calibrated with the 0.9 % TCA solution between each reading.

6) K and Mg in amounts that are even widely above those naturally occurring in bone cause no appreciable interference with the Ca or Na readings.

7) More dilute solutions having the same weight ratios Ca/Na as above give essentially the same findings.

Analyses of bone samples.

Ten samples of a fat free, dry, rabbit bone powder were analysed for Ca and Na content. The samples weighed 100—160 mg and were each vigorously shaken with 3 cc 30 per cent TCA for four hours.

Known amounts of Na and/or Ca were added to five of the bone samples before they were treated with the TCA.

The bone solutions were made up to 100 cc with distilled water before they were introduced in the flame.

Synthetic bone solutions were used as standards. These solutions were of about the same concentrations of Ca, Na, K, Mg and P as those expected in the unknown. The concentration of TCA was kept the same in the standards as in the unknown. The Ca light was read with two standards which emitted more resp. less Ca light than the unknown.

A set of standards was then chosen, having the same Ca emittance as the unknown but with varying Na content. The Na light of the unknown was read with two of these standards, containing more resp. less Na than the unknown.

For each bone sample the lower of the two standard readings was then subtracted from the reading obtained for the unknown. The concentration of Na in the unknown was then obtained by adding to the concentration of Na of the lower standard, the value obtained by interpolating between the two standards.

Table 2.

Analysis for the Calcium and Sodium Content of a Fat Free, Dry Bone Powder.

Sample number	Weight of bone mg	mg Ca per 100 mg bone	mg Na per 100 mg bone
1	122.4	27.6	0.60
2	146.8	28.0	0.57
3	131.7	27.6	0.57
4	150.9	27.3	0.64
5	147.5	28.3	0.57
6	140.9	27.5	0.56
7	111.9	27.0	0.56
8	126.8	28.4	0.53
9	116.6	28.2	0.57
10	104.1	27.3	0.56

Before treatment of the bone samples 6—10 Na and/or Ca were added in the following amounts:

6	0.35	mg	Na				
7	0.71	»	»				
8					12.0	mg	Ca
9					8.0	»	»
10	0.47	»	»	+	8.0	»	»

The result from this series of analyses are presented in table 2. It is seen that the bone powder was found to contain $0.573 \pm \pm 0.0092$ mg Na and 27.7 ± 0.15 mg Ca per 100 mg bone.

These figures correspond with the figures generally cited for the calcium and sodium content of bone (see *inter al.* EDELMAN *et al.* (1952)).

DAVIES and KORNBERG[1] have analysed this bone powder with (1) the method of DAVIES *et al.*, l. c., and (2) direct flame photometry. In this latter procedure the result was corrected for "the apparent sodium light" emitted by a calcium standard containing the same amount of Ca as quoted from analyses made by me as above.

With these methods DAVIES and KORNBERG found the sodium content of the bone powder to be (1) 254 mmol./kg and (2) 248 mmol./kg. Expressed in these terms my analyses above give a sodium content of the bone powder of 250 mmol./kg bone.

It should be mentioned that in routine work the above method makes possible an analysis for the Ca and Na content of 20—30 different samples per hour. Small bones and teeth (*e. g.* from rats) need not be pulverized before shaking with the TCA (see BAUER 1954). Care should be taken to use a long enough shaking time and to use a sufficient amount of TCA solution.

Summary.

The calcium and sodium content of bone dissolved in trichloroacetic acid is determined by flame photometry. The principle is as follows:

1) The concentration of Ca in the unknown is determined by flame photometry.

2) The concentration of Na in the unknown is determined by flame photometry, using as standard a synthetic bone solution. The emittance of Ca light of this standard is that determined in step 1).

This method is accurate and speedy.

Note.

After this method had been elaborated I received a letter from I. S. EDELMAN, San Francisco, USA, informing me that a similar method will be described by him in a forthcoming paper.

[1] I wish to convey my warm thanks to Dr. DAVIES and Dr. KORNBERG of the University of Sheffield, England, for their kindness in making these measurements.

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Experimental Studies of the Human Fetus in Prolonged Asphyxia.

By

GÖRAN ENHÖRNING and BJÖRN WESTIN.

Received 31 January 1954.

The mortality of the newborn during the first twenty-four hours has changed but little during the last decades, due partly to failure in reviving the asphyxiated newborn. In view of this fact, a study has been made of the physiopathology of the asphyxiated human fetus in order to improve the results of resuscitation.

According to X-ray studies of animals (BARCLAY, FRANKLIN and PRICHARD 1945) and of human fetuses (LIND and WEGELIUS 1949), the blood from the umbilical vein goes via the ductus ven. Arantii to the right atrium. From there it proceeds via the foramen ovale to the left heart without mixing to any appreciable degree with venous blood from vena cava cranialis (EVERETT and JOHNSON 1950). It has been suggested that the oxygen and carbon dioxide levels in the blood might influence the closure of the ductus arteriosus by causing changes in the pulmonary vascular resistance (NISELL 1953).

In the asphyxiated newborn, pronounced hypoxemia and hypercapnia have been indicated (EASTMAN 1932, SMITH and KAPLAN 1942). The relative importance of oxygen and carbon dioxide levels, with regard to adaptation to breathing, is not yet established. The tolerance for anoxia in the newborn is high due partly to anaerobic glucolysis (HIMWICH, ALEXANDER and FAZEKAS 1941, BRINKMAN 1953).

Experimental.

The observations we have recorded below are intended primarily to show more closely the effect of oxygenated blood on heart activity, blood pressure and respiratory movements in previable fetuses. Tests were also made to ascertain whether this effect differed from that obtained with venous blood, 5 % glucose or physiol. sodium chloride solution. Finally, the effect of noradrenaline was tested. To be able to judge properly the effects of these different injections, studies of heart activity, blood pressure, and respiratory movements were made in some cases, where no treatment was given.

Method: To register simultaneously the ECG and blood pressure an *Elema Triplex* electrocardiograph (System Elmquist) was used. The galvanometer for lead III was replaced with a pressure-recording device consisting of a capsule. Between the capsule's metal membrane and a fixed point there is a spiral spring equipped with a mirror for reflecting light from the lamp onto the electrocardiographic film. Any variation in pressure within the capsule alters the mirror's position, this in turn shifting the point of light on the film. The arterial blood pressure of the fetus was transferred to the pressure-recording device through a polyethylene catheter inserted in one of the umbilical arteries. In fetuses under 20 cm in length the artery was explored behind the front abdominal wall close to the umbilicus. In larger fetuses the catheter was inserted directly into the artery after previous dilatation with probes.

The calibration curve described a rectilinear course between 0 and 100 cm H_2O. Zero had a fixed position. A variation in pressure of 10 cm H_2O corresponded to 0.87 cm on the ECG film. The measuring tolerance was approximately $\pm$ 0.04 cm on the film, *i. e.* in terms of pressure about $\pm$ 0.5 cm H_2O.

The ECG was registered in lead I, II and IVR. Silverplated clamps were used for electrodes.

The blood introduced was Rh-negative 0-blood. From 3 to 5 cm^3 of blood was injected depending upon the size of the fetus. The blood was oxygenated in a cylindrical, rotating glass container into which oxygen was introduced. There was little scum formation or hemolysis, and sterility was satisfactory.

Material.

The tests were performed on a total of 37 human fetuses, ranging in length from 12—34 cm, obtained both from spontaneous and legally induced abortions. As the statements of patients regarding the length of pregnancy often proved to be incorrect, the *length* of the fetus was considered to be the most reliable gauge of the degree of maturity. The distribution of the material used is shown in table I and II. The legally induced abortions were performed under ether anaesthetic.

Table 1.

Length in cm	Vag. caec. section	Abdom. caec. section	Spontaneous abortions
11—15	1	—	1
16—20	—	4	2
21—25	2	4	6
26—30	—	1	6
31—35	—	2	8
Total number	3	11	23

Table 2.

Length in cm	Untreated Fetuses		Treated Fetuses					
	25° C	37° C	Glucose and Sodium Chloride	Citrate Solution	Noradrenaline	Citrate Blood	Heparin Blood	Perfusion. Hep. Blood
11—15	—	—	—	—	—	1	1	—
16—20	2	—	—	—	—	2	2	—
21—25	—	—	1	1	—	7	1	2
26—30	2	1	—	—	—	4	—	—
31—35	—	2	2	—	2	2	2	—
Total number	4	3	3	1	2	16	6	2

Oxygen was administered to the mother shortly before incision of the uterus was made. In most cases the fetuses were extracted within 5—7 minutes after the anaesthetic was introduced. In those cases where the fetuses were obtained through vaginal hysterotomy, this was done with no difficulty and without injury to the fetuses.

The ECG and blood pressure were recorded continuously and not terminated until signs of heart activity on the ECG ceased. This occurred in the majority of cases between the third and fourth hour after delivery. Recordings were made at a temperature of 25° C.

Results.

I. Untreated Fetuses.

The ECG in abdominally delivered fetuses.

The P-wave was present and positive in all cases in all leads.

The P—Q-time 3—10 minutes after delivery and regardless of the length of the fetus was 0.06—0.08 second. Within 15—30 minutes the P—Q-time was lengthened to 0.10 second. The con-

duction time was lengthened to 0.12 second at the earliest after 25 minutes.

The *S—T level* was isoelectric in all cases. A decline under the isoelectric line occurred at the earliest about 45 minutes after delivery.

The *T-wave* was clearly marked and positive in all leads 3—10 minutes after delivery. The height of T_1 3—10 minutes after delivery was 1.5 ± 0.5 mm. The height then declined quickly and was negative after 45—60 minutes.

Right preponderance was present in all cases.

The rhythm was regular; irregularities in the form of extra systoles were advanced changes.

The *heart rate* recorded after 3—5 minutes reached a maximum of about 120/minute. The rate then declined sharply and was about 25—35/minute after 45 minutes.

The ECG in spontaneous abortions.

In principle the fetuses showed similar changes on the ECG but the heart rate was often lower at birth than in the legally induced abortions. The changes recorded on the ECG usually occurred earlier and were more pronounced.

The blood pressure.

Twenty minutes after birth, at a heart rate of about 100/minute, the blood pressure of fetuses of about 30 cm in length was 70—72 cm H_2O. Corresponding values for fetuses about 20 cm long, at a heart rate of 70/minute, were 28—30 cm H_2O. After 45 minutes the blood pressure in both groups was between 15—25 cm H_2O.

The steady decline in the heart rate and blood pressure and the rising conduction time are recorded in fig. 1.

Changes in excitability.

In fetuses born within the membranes, no limb or respiratory movements were observed as long as the fetuses remained within the ovum. If the membranes were opened a few minutes after birth, the fetus immediately made a few spontaneous movements of the limbs. These reflexes were also possible to cause by touching any part of the fetus. About 10 minutes after birth these reflexes could no longer be produced.

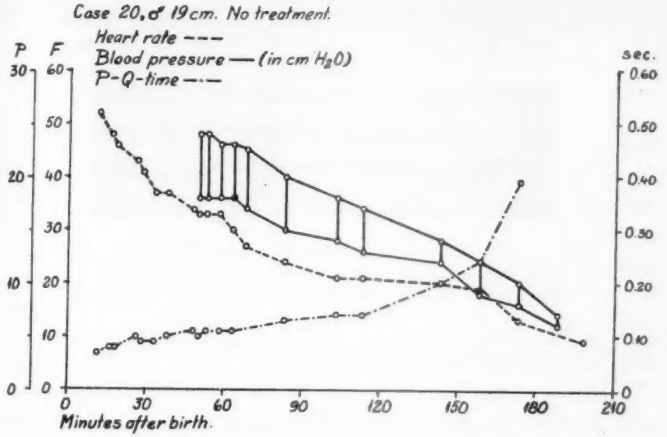

Fig. 1. Legally induced abortion.
P = Blood pressure.
F = Heart frequency.

Spontaneous gasps.

Repeated spontaneous gasps occurred in only one of the induced abortions.

The respiratory movements came in series of 2—3 gasps at intervals of a few minutes and continued for 30 minutes. In one spontaneous abortion repeated spontaneous gasps occurred during $2^1/_2$ hours. In both these cases the lungs were atelectatic and no air in the gastro-intestinal canal could be detected after death.

In both cases a temporary decline in blood pressure was registered during inspiration. At the same time, owing to the change in the intrathoracal pressure, blood was sucked from the umbilical vein into the vena cava caud. This could be clearly seen in the catheter inserted into the umbilical vein. The change in arterial blood pressure under the influence of these respiratory movements is shown in fig. 2.

The heart rate and blood pressure in both these cases were about twice as high as in the other cases as late as 45 minutes after birth. The presence of gasps in these fetuses perhaps point to a higher degree of maturity, but it is obvious on the other hand that the breathing movements in themselves improved the circulation by accelerating the venous return to the heart.

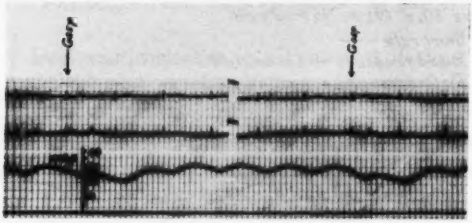

Fig. 2. Spontaneous abortion (male, 29 cm).
ECG recorded in lead I and II 54 minutes after birth.
Note decline in blood pressure when gasps are performed.

The influence of temperature on survival time.

In three cases (case 35, male 34 cm; case 36, female 30 cm; case 37, male 27 cm) the survival time was recorded at a temperature of 37° C until signs of heart activity ceased. The three foregoing cases survived 65, 92 and 90 minutes, respectively. In these cases the survival time was much shorter compared to the recordings made at a temperature of 25° C.

II. Treated Fetuses.

The fluids injected had a temperature of 25° C and the doses ranged from 3—5 cm³ depending on the size of the fetus. (By oxygenated blood is meant blood saturated with oxygen.)

Citrate blood[1] *injected in the umbilical vein.*

The introduction of *oxygenated* blood caused a rapid increase in heart rate and blood pressure in the asphyxiated fetus. Negative T-waves became positive and low T-waves rose higher. A temporary increase in conduction time was also recorded. Gasps were produced.

When venous blood was injected blood pressure and heart rate increased only imperceptibly and the height of T_1 was not significantly altered. The introduction of venous blood moreover caused a permanent increase in conduction time.

The effect of *oxygenated* and *venous* citrate blood on heart rate,

[1] Citrate Blood:
ACEDEX 65 cm³
Blood 350 »

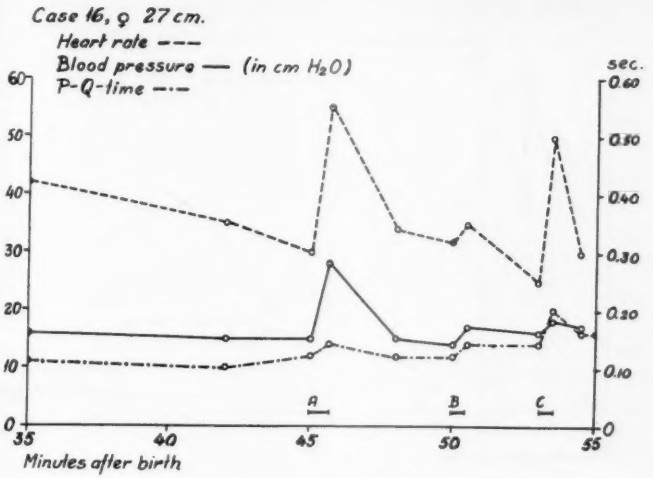

Fig. 3. A = 3 cm³ oxygenated citrate blood.
B = 3 » venous citrate blood.
C = 3 » oxygenated citrate blood.

conduction-time and blood pressure is shown in fig. 3. Injections A and C in this figure show the rise in blood pressure and heart rate, when oxygenated blood was injected. The T_1-wave raised sharply but deviated from normal both with respect to height and breadth. The S—T-interval was lengthened compared to that for untreated fetuses with comparable heart rate. Injection B shows the insignificant effect on blood pressure and heart rate when venous blood was injected. A lengthening of conduction time was noted.

5 % glucose or physiol. sodium chloride solution in the umbilical vein.

These fluids injected into the umbilical vein caused only a slight increase in heart rate and blood pressure. A small lengthening of the P—Q-time was simultaneously recorded. The effect of 5 cm³ of glucose solution compared to that of 5 cm³ oxygenated citrate blood is seen in fig. 4. At A and C oxygenated blood was injected. At B the same amount of glucose solution was injected at the same speed as injection A. The insignificant effect of glucose on heart rate and the lengthening of conduction time were noted. Repeated gasps lasting 8 minutes were produced by injection C.

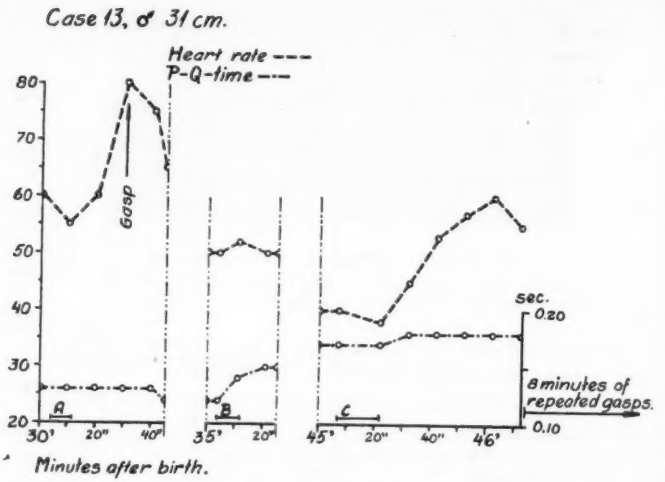

Fig. 4. A = 5 cm³ oxygenated citrate blood.
B = 5 » 5 % glucose solution.
C = 5 » oxygenated citrate blood.

Single injections of glucose or sodium chloride solution did not produce gasps.

Heparin blood in the umbilical vein.

The blood specimens were drawn directly from the cubital vein of an adult. The syringe was first moistened with heparin solution. No glucose was added.

Venous heparin blood proved to have an effect on the ECG and blood pressure comparable with that produced by oxygenated citrate blood. Heparin blood, however, did not lengthen conduction time and the T-waves assumed normal configuration.

Oxygenated heparin blood had a far better effect. The results obtained from such an injection about $1^1/_2$ hours after birth are shown in fig. 5. A very sharp increase in heart rate and blood pressure approaching normal value was attained. The ECG was normalized. Heparin blood also proved capable of producing gasps. The effect of oxygenated heparin blood compared with that obtained from oxygenated citrate blood is shown in fig 6. The difference in configuration of S—T intervals and T-waves is noted.

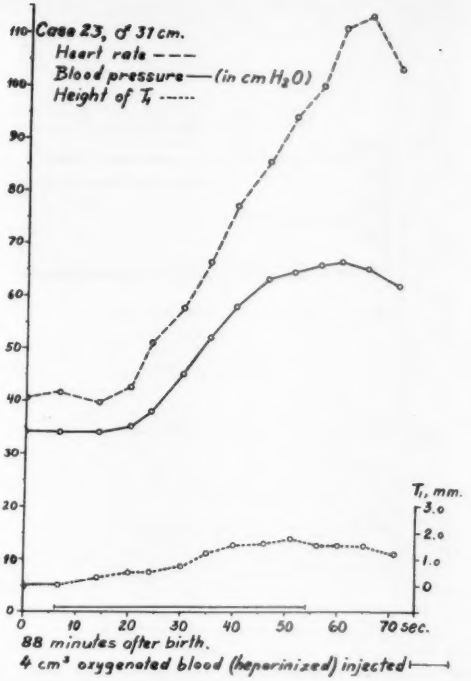

Fig. 5. Normalization of heart rate and blood pressure. Height of T_1 within normal limits when oxygenated heparin blood was injected.

Citrate solution in the umbilical vein.

65 cm³ of ACEDEX[1] solution was diluted with 350 cm³ of distilled water. Five cm³ of this solution was injected during 35 seconds (case 32). In two other comparable fetuses the same amount of *oxygenated* citrate (case 33) and heparin (case 34) blood was injected at the same speed and after the same time lapse following birth. Being single doses, the volumes injected were large considering the size of the fetuses and the injection speed. The

[1] ACEDEX.

Natrii citras tribasicus	2.2 g
Acidum citricum	0.8 »
Glucosum	2.5 »
Aqua steril.	ad 100 cm³

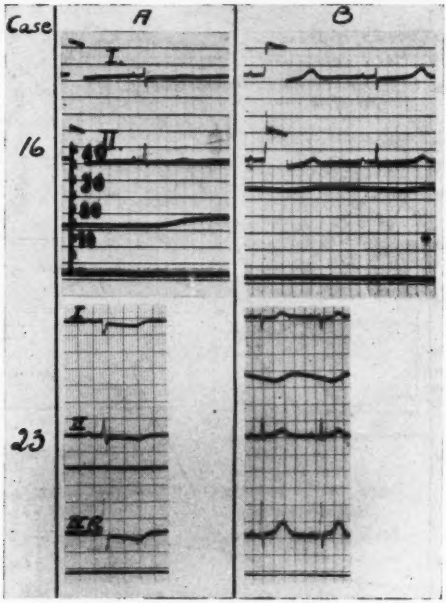

Fig. 6. Legally induced abortions.
A = Injection started. B = Injection ended.
Case 16: 3 cm³ oxygenated citrate blood 45 minutes after birth.
Case 23: 4 cm³ oxygenated heparin blood 88 minutes after birth.
Note difference in length of S—T interval and configuration of T-waves at B.

effect on heart rate and survival time is shown in fig. 7. On injecting pure citrate solution, the fetus assumed an opistotonus position and a tonic cramp of short duration occurred in the limbs. In connection herewith the usual signs of heart activity on the ECG ceased for 6.5 seconds and were replaced by frequent, low waves on the isoelectric line (auricular fibrillation). Thereafter the ventricular complexes reappeared. The S—T-intervals were lengthened and the T-waves became broad and very pronounced. Repeated extrasystoles followed whereupon signs of heart activity on the ECG ceased two minutes after the injection was begun. — The relatively large volume of citrate blood quickly gave in this case a pathological ECG with extrasystoles and short survival time. The S—T-intervals and T-waves were similar to those produced by the citrate solution. Despite the fact that the

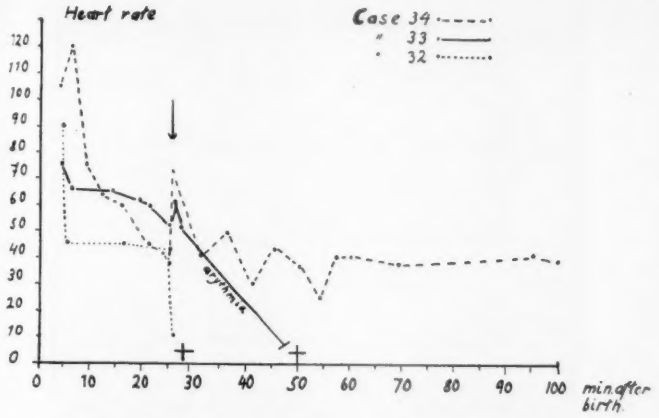

Fig. 7. Legally induced abortions. Injections started at arrow.
+ = Signs of heart activity on the ECG ceased.
Case 32 (male, 23 cm): 5 cm³ citrate solution (+).
Case 33 (female, 22 cm): 5 cm³ oxygenated citrate blood (+).
Case 34 (male, 18 cm): 5 cm³ oxygenated heparin blood.

fetus given heparin blood was smallest, no perceptible changes were recorded on the ECG (fig. 8).

Large doses of citrate solution or citrate blood under asphyctic conditions have a deleterious effect on heart activity.

Single injections of noradrenaline.

The effect of 0.05 mg noradrenaline in 1 cm³ of glucose solution injected into the umbilical vein was studied. A moderate rise in blood pressure was noted. Heart rate rose but little. Pronounced pathological changes in the ECG occurred within 30 seconds. These changes were similar to those found in adults in coronary thrombosis of the anterior wall of the heart. Gasps were not produced.

Oxygenated heparin blood in the umbilical arteries.

In agonal states of the fetuses with blood pressure at zero, pronounced pathological, but regular ventricular complexes in the ECG were the only signs of life. — In these cases, oxygenated heparin blood injected into the umbilical *vein* failed to restore heart activity and blood pressure.

The rapid injection of 5—10 cm³ oxygenated heparin blood into one of the umbilical *arteries* under simultaneous compression of

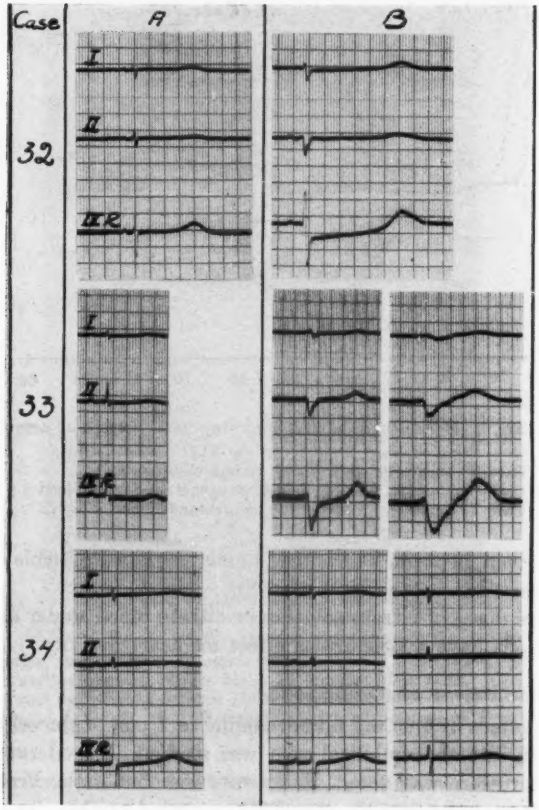

Fig. 8. Twenty-five minutes after birth.
A = Before injections.
Case 32. B = 1 minute after citrate solution.
Case 33. B = 5 resp. 8 minutes after oxygenated citrate blood.
Case 34. B = 1 resp. 25 minutes after oxygenated heparin blood.

the femoral arteries improved the circulation within 30 seconds. A rapid increase in heart rate with vigorous pulsations of the praecordium was noted. The pathological changes in the ECG improved and blood pressure was maintained at a high level for several minutes. When releasing the compression of the femoral arteries, the pink colour of the cranial half of the body suddenly flushed over the pale and cyanotic, caudal part of the body and the legs.

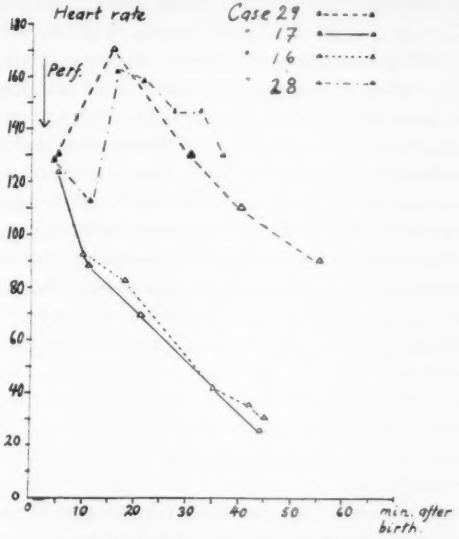

Fig. 9. Legally induced abortions.
Perfusion with oxygenated heparin blood at a temperature of 37° C started at arrow.
(Case 28, male 21 cm and case 29, male 24 cm.)
Controls recorded at a temperature of 25° C.
(Case 16, female, 27 cm and case 17, male 20 cm.)

Perfusion with oxygenated heparin blood.

Perfusion tests on animal fetuses have previously been conducted by THOMAS (1948), but they have not been described on human fetuses.

Oxygenated heparin blood was introduced into the human fetus by a catheter inserted into the umbilical vein under a pressure of about 10 cm H_2O. The fetus was placed in a solution of equal amounts of *Macrodex* and *Ringer*-glucose solution with a temperature of 37° C. Catheters were then inserted into both umbilical arteries through which the blood was pumped into a container. Thereupon this blood was oxygenated and transferred to the container for the umbilical vein. By regulating the height of the two containers above the heart of the fetus, the balance between output and input could largely be maintained. The results of these preliminary perfusions (cases 28 and 29) are shown in fig. 9, where heart rate is compared with that of two untreated

fetuses (cases 16 and 17). In case 28 the blood was obtained from the brachial artery of a healthy adult. Fifteen minutes after delivery, blood specimens were drawn from one of the umbilical *arteries* of the fetus. The oxygen tension was 44 mm Hg. The blood pressure at that moment was 40 cm H_2O. — A few minutes after the perfusions were commenced, the fetuses produced a few gasps. Thereafter they were quiet with the exception of single, insignificant movements of the limbs. However, they were extremely excitable to touch which incited vigorous limb movements. After about an hour there was a marked tendency of extravascular haemorrhages which shortly afterwards caused death.

A special apparatus for the perfusion of human fetuses has been designed and the experiences gained from its use will be published later.

Discussion.

With respect to the fetuses studied by means of the ECG and blood pressure we have noted indications of progressing asphyxia, such as continuous lowering of the heart rate, disturbances in conduction time, lowered or negative T-waves as well as a steady decline in blood pressure.

Most of these conditions are quickly eliminated after treatment with moderate doses of oxygenated *citrate* blood. In addition to oxygen, however, calcium-binding citrate ions were also introduced, the benefit of which may still be debated.

In substituting the blood of newborn infants, BRANCATO (1950) earlier stressed the danger of resulting hypocalcemia.

The removal of calcium from blood and serum by *Amberlite* (cat-ion exchanger) in the presence of sodium citrate is incomplete. On the other hand, heparin has no effect on the adsorption of calcium by *Amberlite* (HUSSEY and al. 1950). In other words, heparin has no calcium-binding power.

But, low and even negative T-waves are believed to occur in cases of hypocalcemia (LJUNG 1949). According to OSNES (1951), anoxia is accompanied by a cell-dehydration with sodium replacing a large portion of the intracellular potassium. In conserved citrate blood, the blood corpuscles lose potassium to the serum. This process is accelerated if the blood is heated (SCHWALM 1952). The effect of potassium on the ECG in healthy dogs is

increased amplitude of T_1 and a lowered S—T-level (BELLET and GAZES 1950).

Thus, it would seem that an injection of citrate blood into the asphyxiated fetus could cause changes in the ECG difficult to explain. The introduction of oxygenated citrate blood always produced a marked rise of the T-waves. This is believed to be caused partly by the introduction of oxygen. This assumption is supported by the fact that venous citrate blood did not appreciably change the height of the T-waves and it, furthermore, caused a permanent lengthening of conduction time. It is probable that hypoxia is mainly responsible for this change. On the other hand, venous and oxygenated citrate blood all gave rise to a marked broadening of the T-waves and a lengthening of the S—T intervals. This was also the case when citrate solution was injected. As the citrate solution did not contain any potassium ions, hypocalcemia was the most probable cause of the effect on the ECG and of the heart standstill. The presence of tetanic cramps of the fetus during the injection also points in that direction. Thus, hypocalcemia is also the probable cause of the deleterious effect on asphyxiated fetuses of large doses of citrate blood. In consequence thereof, the introduction of large doses of citrate blood in conditions of severe asphyxia seems to be contra-indicated. In this case, heparin blood should instead be used to eliminate the danger of hypocalcemia.

The effect of noradrenaline on the heart is of great interest. There is good reason to assume that the vasodilatation caused by the freeing of adrenaline and noradrenaline is, at least partly, secondary to the increase in metabolism of the heart muscle cells induced by these substances. An increase in blood supply should therefore be adapted to an increase in metabolism. Hence, such substances as noradrenaline are not suitable to be used for improving nutrition of the myocardium and for eliminating a relative ischemia (FOLKOW 1953). Even if the noradrenaline doses we have used are large, its effect on asphyxiated fetuses with quickly occurring pathological changes in the ECG is in agreement with this opinion.

The effect of oxygenated heparin blood on heart rate, blood pressure and primitive respiration in the human fetus would seem to indicate its value in reviving newborn infants. Clinical tests proved that viable deeply asphyxiated newborn infants could be revived by means of transfusion with oxygenated blood where the

customary treatment produced no effect. Perfusion with oxygenated blood may be of value in the treatment of premature infants.

Summary.

A total of 37 previable fetuses was studied with particular emphasis on changes in the ECG and blood pressure under increasing asphyxia:

1) In *untreated* fetuses, the recordings showed a steady decline in blood pressure and heart rate, while conduction time increased. Positive T-waves become negative in lead I, II and IVR. The arterial blood pressure dropped temporarily when gasps were produced by the fetus. With each gasp a negative pressure in the thorax was established, accelerating the venous return to the heart. Temperature influenced survival time.

2) The introduction of *oxygenated heparin blood* into the umbilical vein caused a rapid and sharp rise in heart rate and arterial blood pressure. The very rapid normalization of the ECG supports the contention that oxygenated blood introduced into the umbilical vein quickly reaches the left atrium and thus the coronary arteries. The respiratory centre appeared able to withstand protracted asphyxia. The intra-venous introduction of oxygenated blood caused repeated gasps as late as 45 minutes after birth. In agonal states of the fetus oxygenated heparin blood had to be introduced through one of the umbilical arteries in order to improve circulation. The perfusion of previable fetuses with oxygenated heparin blood is described.

3) Large doses of *citrate or citrate blood* brought about pathological changes in the ECG and shortened survival time, due probably to hypocalcemia.

4) Noradrenaline gave rise to pathological changes in the ECG of the asphyxiated fetus.

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Prothrombin Times of Mixtures of Various Types of Plasmas with Lowered Prothrombin Activity.

By

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Several publications from this laboratory (DAM and SØNDERGAARD 1948, 1953 b, SØRBYE, KRUSE and DAM 1950 a, 1950 b have dealt with the fact that mixtures of plasmas from vitamin K-free chicks and chicks treated with anticoagulants of the dicoumarol type, under suitable conditions have a shorter prothrombin time than the individual plasmas of the mixture.[1]

The work to be reported here shows: 1. that other anticoagulants of the dicoumarol type than those previously tested behave similarly, 2. that under certain conditions such plasma mixtures do not show a depression of prothrombin time, 3. that mixtures of two plasmas from chicks treated in different ways with the same anticoagulant may show slight depression of prothrombin time, 4. that a more pronounced depression of prothrombin time can be obtained with mixtures of plasmas from chicks treated with two different anticoagulants, and 5. that addition of chicken serum prepared under varied conditions lowers the prothrombin time of mixtures of coumachlor plasma and vitamin K deficient plasma and may eliminate the depression.

Experimental.

The experimental procedure was the same as described in the papers already cited. The prothrombin times were determined as by DAM,

[1] This phenomenon has been confirmed by J. MORAUX (1951) working with chicks, vitamin K deficiency, dicoumarol, phenylindanedione and diphthiocol.

KRUSE and SØNDERGAARD (1951); the diets used were those described by DAM and SØNDERGAARD (1953 a).

Results.

The results are presented in figs. 1 to 5.

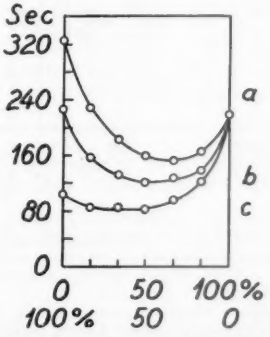

Fig. 1. The depressions obtained by mixing one and the same vitamin K-free plasma with three different plasmas from chicks treated with 3-(alpha-p-nitrophenyl-beta-acetylethyl)-4-hydroxy coumarin (a), 2-p-chlorphenyl-1,3-indanedione (b), and 2-(diphenylacetyl)-1,3-indanedione (Dipaxin) (c), respectively. Compound a was given on two consecutive days, 0.05 mg per g body weight per day, the blood collected on the third day. Compounds b and c were given as single doses, 0.5 mg per g body weight, the blood collected two days after dosage.
Ordinate: Prothrombin time.
Abscissa: Vitamin K-free plasma right,
Anticoagulant-plasma left.

Discussion.

The cause of the depression of prothrombin time in mixtures of two different plasmas must be that each of the two plasmas contains a factor which is diminished in the other. The reason why optimal mixtures do not show normal prothrombin times could be that both plasmas are deficient in a common third factor (DAM and SØNDERGAARD 1948), or more or less deficient in the two first mentioned factors.

The factor contained in vitamin K deficient plasma and lacking in dicoumarol plasma has been arbitrarily termed the *kappa factor*, whereas the factor contained in dicoumarol plasma and lacking in vitamin K deficient plasma was termed the *delta factor* (SØRBYE, KRUSE and DAM 1950 a, b). These two factors were

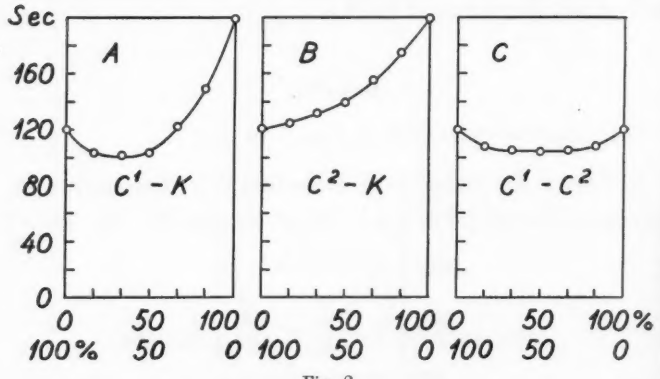

Fig. 2.

A: The usual depression of prothrombin time obtained when vitamin K-free plasma (right) is mixed with plasma from a chick treated with coumachlor given in doses of 0.05 mg per g body weight on two consecutive days and the blood collected on the third day.

B: The absence of depression when the same vitamin K-free plasma (right) is mixed with plasma from a chick treated with coumachlor as in A with the only difference that the blood is collected on the fourth day.

C: The slight depression obtained when the two coumachlor plasmas referred to in A and B are mixed with each other.

Ordinate: Prothrombin time, sec.

Abscissa: Concentrations of the plasmas in the mixtures.

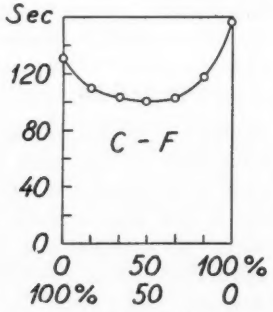

Fig. 3. The pronounced depression obtained by mixing plasma from a chick treated with coumachlor (right) given in doses of 0.05 mg per g body weight on two consecutive days, the blood collected on the fourth day as in fig. 2 B, with plasma from a chick treated with one dose of compound b (fig. 1), 0.5 mg per g body weight, the blood collected on the third day as in fig. 1.

Ordinate: Prothrombin time, sec.

Abscissa: Concentrations of the plasmas in the mixtures.

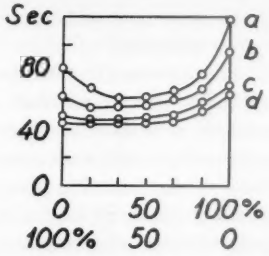

Fig. 4. The effect of adding 0.1 ml serum to 0.6 ml of a mixture of vitamin K-free plasma (right) and coumachlor plasma prepared as in fig. 2 A.
Curve a: No serum added.
Curve b: Addition of serum prepared by adding 2 drops of undiluted thromboplastin solution to 8 ml blood taken from the carotid artery of a normal chick, leaving the clotted blood over night at 15° C, centrifugation and addition of 0.025 ml 2.76 % potassium oxalate (1 mol. H_2O) per 1 ml serum so as to precipitate the calcium present.
Curve c: Addition of serum prepared similarly as in b but with thromboplastin diluted with 2 vol. 0.9 % NaCl solution.
Curve d: Addition of serum prepared with thromboplastin diluted with 9 vol. 0.9 % NaCl solution.

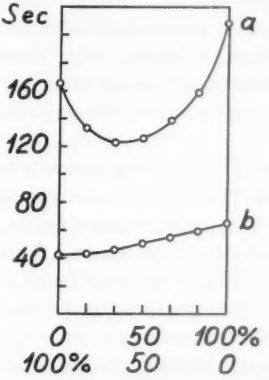

Fig. 5. The effect of adding 0.1 ml serum to 0.6 ml of a mixture of vitamin K-free plasma (right) and coumachlor plasma (left) prepared as in fig. 2 A.
Curve a, no serum added.
Curve b, addition of serum obtained by spontaneous coagulation of blood from a normal chick.

adsorbed from the respective plasmas on barium carbonate, eluted with citrate and their effect on the other plasma, resp. the ineffectiveness against the same plasma was demonstrated.

Since criteria on the purity of the kappa and delta factors are

not available it could be that each of them represents a relative excess of one factor over another.

Such a view would be in agreement with the findings of MANN, MANN and BOLLMAN (1950) who found that, under given conditions, in rats, dicoumarol decreased co-thromboplastin activity much more than prothrombin, while vitamin K deficiency produced a greater decrease in prothrombin than in co-thromboplastin. Co-thromboplastin is believed by several investigators (ALEXANDER, FLYNN, MANN, OWREN and SEEGERS 1952) to be identical with proconvertin of OWREN (1951).

A predominant decrease of proconvertin due to dicoumarol treatment has also been found by OWREN and AAS (1951) and by KOLLER et al. (factor VII) (1951).

From this point of view the kappa factor should represent an excess of proconvertin over prothrombin, and the delta factor an excess of prothrombin over proconvertin. Since mixtures of the two plasmas do not show normal prothrombin times it might be inferred that both plasmas contain subnormal amounts of proconvertin and prothrombin. It would then be expected that simultaneous addition of kappa and delta factors to plasma mixtures showing a depression would bring about a decrease also of the minimum prothrombin time of the plasma mixtures. However, this was not found to be the case (SØRBYE, KRUSE and DAM 1950 b).

Since the chemical unity of the factor termed proconvertin is not certain either, the final solution of these questions will require further experimentation. Such studies are in preparation.

In any case, it is obvious that vitamin K is concerned with the formation of all the factors involved in dicoumarol intoxication. Otherwise it would be impossible to obtain normalization of dicoumarol plasma with vitamin K_1.

In the present experiment it was possible to select the conditions in such a way that either the depression of prothrombin times of mixtures of vitamin K deficient plasma and plasma from anticoagulant-treated chicks was eliminated, fig. 2 B, or a depression was obtained with two plasmas from chicks treated with the same anticoagulant but in different ways, fig. 2 C. In both cases the results must be explained by differences in the rate of disappearance, resp. of resynthesis of plasmatic factors.

QUICK et al. (1952) failed to find a depression of prothrombin time when plasmas from cholecystonephrostomized dogs were

mixed with plasma from dicoumarol treated dogs. The reason for this is not clear. It could be that either the prolongation of prothrombin times were not sufficiently marked to allow the finding of a depression of more than 1.2 sec. as indicated by these authors, or that the conditions with respect to dicoumarol treatment have been similar to those in our present experiments, fig. 2 B, where no depression occurred.

In our experiments, fig. 4, serum prepared in various ways was added to mixtures of coumachlor- and vitamin K-free plasmas. Serum prepared with the lowest concentration of thromboplastin caused the most marked lowering of the prothrombin times of the plasma mixtures (curve d). The lowering was particularly marked for coumachlor plasma or the mixtures consisting mostly of coumachlor plasma, so that the curve became horizontal at the "coumachlor end". This must mean that the serum corresponding to curve d has been especially rich in factors (particularly the kappa factor) lacking in the coumachlor plasma. The serum has also contained a certain amount of the factors lacking in vitamin K-free plasma or the factor lacking in both ("the common factor"). It cannot have contained much of the delta factor since otherwise it would have straightened the curve out at the "vitamin K-free end". It is likely therefore, that the serum added in experiment d has contained some of a factor lacking in vitamin K-free as well as in coumachlor plasma.

In fig. 5 b, where serum obtained by spontaneous clotting was added to mixtures of vitamin K-free and coumachlor plasma the curve was further straightened out, suggesting that the factors lacking at both ends of curve a have been present in the serum.

In certain experiments by OWREN (1952) it was found that in human serum, obtained by spontaneous coagulation, prothrombin disappeared on standing for several hours while proconvertin remained constant. When thromboplastin (dilute) was added, prothrombin disappeared even more rapidly. According to the function ascribed to proconvertin it must be assumed that with increasing amounts of thromboplastin added less proconvertin will remain in the serum. If these considerations can be applied to our experiments it will mean that the serum used in d (fig. 4) has contained more proconvertin than the serum in b. In that case it could be assumed that the kappa factor is or mainly is proconvertin. However, the serum used in our experiments, especially fig. 5 b, has contained at least one more factor than

the kappa factor; therefore, further studies are required to solve the problem.

Summary.

The shortening of the prothrombin time obtained by mixing plasmas of a vitamin K deficient chick with plasma from chicks treated with dicoumarol or coumachlor has been demonstrated also with three other anticoagulants, viz.: 3-(alpha-p-nitrophenyl-beta-acetylethyl)-4-hydroxycoumarin, 2-p-chlorphenyl-1,3-indanedione and 2-(diphenylacetyl)-1,3-indanedione (Dipaxin).

Variation of the experimental conditions (prolongation of the time interval between administration of the anticoagulant and the collection of the blood sample) may result in elimination of the shortening of the prothrombin time when the plasma is mixed with plasma from a vitamin K deficient chick.

Mixtures of plasmas from chicks treated with the same anticoagulant but with different time intervals from administration of the anticoagulant to the collection of the blood sample may show a slight shortening of the prothrombin time.

Addition of chicken serum stored for 20 hours, to mixtures of coumachlor- and vitamin K deficient plasmas causes a lowering of the prothrombin time curve. This effect is particularly marked at that end of the curve which is near 100 per cent coumachlor plasma. When sufficiently dilute thromboplastin solutions have been used for preparation of the serum added, the prothrombin time curve becomes almost horizontal in the region from 50 to 100 per cent of coumachlor plasma. This observation is discussed in relation to certain experiments of other investigators.

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Formation and Elimination of CO in Mammals.

By

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It has been shown in several investigations that CO can be oxidised by the skeletal and heart muscle tissues (FENN and COBB 1932, STANNARD 1940, CLARK, STANNARD and FENN 1950, BRECKENRIDGE 1953). In incubated blood to which CO was added, SJÖSTRAND found (1951) that CO concentration decreased if the original COHb concentration exceeded 5 %. With lower COHb concentrations, on the other hand, there was found an increase (SJÖSTRAND 1951, 1952).

In investigations on turtles and mice, which were exposed to CO in a tightly closed chamber for several days, CLARK (1950) found that the CO concentration decreased which he interpreted as being due to oxidation of CO in the organism. The same result appeared also in experiments when radioactively labelled CO was used, where an absorption of C^{14} by the CO_2 absorber could be demonstrated. The amount of CO oxidation calculated from these experiments was essentially greater than expected when taking into consideration the rate of oxidation in the muscle tissue.

In a series of investigations on humans, rabbits and dogs an intravital formation of CO was demonstrated which can be explained as due to a liberation of CO by the decomposition of hemoglobin (SJÖSTRAND 1949, 1951, 1952). From these different observations it seems that CO can be formed as well as oxidised in the organism in the same way as in blood solutions.

In the present paper the results of an investigation are reported, where Clark's experiments have been repeated using

guinea pigs and rabbits. These were kept in a chamber, both with and without addition of CO to different concentrations.

Method.

Apparatus.

Different devices for exposing animals in the chamber to a certain CO concentration were tried but rejected as they gave unreliable results. Thus a metal chamber has been used which was shown even in the absence of the animal to give a relatively large decrease of CO concentration even though all leakage had been excluded. Different experiments with circulation of gas in the chamber have been abandoned because even they showed a large CO loss. Among other things it has been found necessary to try to avoid the use of rubber tubing and to pass the gas over water or oil surfaces. The following arrangement has in the main given definite and reproductive results.

The animals have been placed in a glass bottle of 25 lit. capacity. So that the animals can be placed in the bottle easily and in order to clean it which has been shown to be of great importance, the flask has been divided into two parts, an upper and a lower which can easily be joined together with grease and held in place by weights applied to the upper part. When the animals have been placed in the bottle and both portions approximated, the flask has been filled with 100 % oxygen or air. By means of a pump the gases have been kept circulating through a filter of NaOH for absorption of CO_2. In the circulatory tubing an opening has been arranged for the injection of CO which was closed with a rubber plug. Injection of the gas has been performed with a paraffined record syringe. Inside the glass bottle a rubber bag was placed with a connection to the exterior for pressure adjustment in connection with CO_2 absorption and taking tests. Since 5 lit. of gas were required for an exact analysis of the CO concentration and since it was a desideratum that the chamber be made as small as possible, for every testing, one liter of gas from the flask was diluted with 4 liters of CO free air with the aid of a membrane pump. This has given a constant air flow by means of which the selected volume could be determined from the time of sampling; determined with a chronometer. The coincident airflow to the rubber bag within the chamber due to pressure decrease has been measured by means of a gas meter. Direct sampling via the gas meter has given unreliable values, partly because of the proportionately large dead space of the meter and partly, probably, because the gas in the meter was in contact with water, in which CO could be absorbed.

For the control of complete pressure equalisation in the circulatory system, a Hg manometer has been connected to it and this could be brought into use by means of a stopcock. The manometer has likewise been used for controlling the tightness of the system both before and

after the experiment by an evoked increase or decrease of the pressure in the chamber.

CC concentration has been determined in a LINDELÖV-SJÖSTRAND CO analyser (SJÖSTRAND 1944, 1948). CO free air has been supplied to replace the air which has passed through the analyser or a hopcalite filter. The air has been controlled in the analyser.

In experiments on rabbits, O_2 has been supplied continuously via a Demand air regulator to make up for the volume consumed by the animal. The regulator consisted of a flask partly filled with liquid paraffin, in which two glass tubes had been inserted, one of such a length that it reached one half mm below the paraffin surface. The air regulator replaced the rubber bag inside the flask which in the guinea pig experiments facilitated pressure adjustment.

Mode of Procedure.

The animals, guinea pigs and rabbits, have been subjected to the experiments in the morning after the usual feeding. The flask has always been well cleaned before every experiment. Fresh caustic soda has been used each time. The pump velocity has been controlled with a gas meter for 10 minutes. After this the animals have been placed in the flask which has been sealed completely and for one hour the circulating system has been flushed with 99 % O_2. Following this the supply of O_2 has been shut off and the pressure reduced to 20 mm Hg by aspiration of O_2. For 30 minutes a constant pressure has been maintained according to the manometer. After this a corresponding increase of pressure has been produced and maintained for a further 30 minutes. Two gas samples have been taken in order to control the testing and pressure equilibrium. Subsequently O_2 has been supplied to equalise the pressure. Afterwards CO has been supplied by injection into the circulating system and the pumping system has been incorporated. At the end of two hours two samples have been taken for analysis: this period of time proved sufficient for the attainment of equilibrium between the animals COHb concentration and CO concentration in the glass bottle. Two more gas samples have been taken after a further 10 hours (in the case of the rabbit experiments) or 12 hours (in the case of the guinea pig experiments).

The same experiments have been performed in the absence of the animal, where urine as well as faeces have been left in the chamber. Not less than 48 hours after, the experiments have been repeated on the same animal.

In calculating the CO volume after a period of observation of 2, 12 and 14 hours, respectively, it has been necessary to take into consideration the total volume of gas in the flask. In the guinea pig experiment this has varied while it has been constant in the rabbit experiments. In calculating the eventual amount of CO oxidation in the animal organism the elimination of CO in the experiments has been reduced by the same amount as in the empty chamber. The latter CO

volume has varied, at a concentration of 0.05 % CO, between 0.0125 and 0.05 ml of CO during experimental periods of from 10 to 12 hours.

Accuracy of the Determinations.

The chief error of the calculations are due to errors by the sampling and the determination of the CO concentration in the CO analyser. By determination of the volume of the gas samples as well from the sampling time as from the reading of the gas meter the errors in sampling and dilution have been cut down to around 15 ml.

The CO analyser facilitates a determination of the CO concentration down to 2 parts of CO in 10^7 parts of air or O_2. Using a magnifying glass and millimeter scale and observance of a constant room temperature, it is possible to increase the sensitivity somewhat. The determination of the CO concentrations in question in these experiments, has been accomplished with an accuracy of $\pm$ 1.5 to 3 % of the value. The accuracy is greater with high than with low concentrations of CO. By taking duplicate samples and making full use of average values in the calculations, the accuracy has been still further increased.

An essentially greater number of determination have been made than are reviewed below. These determinations not included, have been rejected because of the difficulty of fixing the optimum initial concentration of CO or because of the accidental unreliability of the CO analyser. Only those values have been included where the discrepancy between the determination on a standard gas composition did not exceed 0.2 mm (0.00004 % CO) in three analyses in serie.

Errors of the Method.

With experiments of the type mentioned a completely airtight apparatus is of course a *conditio sine qua non*. By control of the increased and decreased pressures respectively both before and after the experiment, it seems that this possibility of error has been eliminated. The air tightness of the apparatus has also been checked now and then by following the pressure in the system with increased and decreased pressures respectively in the absence of the animal, during double the time the experiment with the animal took.

To cut out diffusion through the tubing, vacuum tubing has been used through which glass channels have been run. As far as is possible the action of fresh water has been excluded from the system. It has not been possible to eliminate small amounts of urine and feaces but this should not be of any great importance since it must be assumed that an equilibrium between the CO concentration in the chamber and in the whole of the animal body occurs within the first two hours.

It has been shown that animals subjected to a longer experimental period develop symptoms of poisoning in the form of convulsions and paralysis. In the experiment with CO in O_2, this must be due to O_2

poisoning. Using higher concentrations of CO than 0.05 %, as in the case of CLARK who used 0.07 %, this has been more usual. On doing this a combination of O_2 and CO poisoning should appear. In the experiments with CO in air symptoms of CO poisoning appear corresponding to the concentration. In isolated cases the animal has shown symptoms of poisoning without the application of CO and this cannot be explained. Since these different poisonings might be expected to influence the result, the experimental period and CO concentration have been selected so that poisoning symptoms appeared in exceptional cases only. When the symptoms have been more marked the experiment has been rejected.

Table 1.

Increase of the CO-volume in an airtight chamber with guinea pigs and rabbits during 12 and 10 hours stay respectively after a 2 hours equilibration period.

No.	Animal	Body wt g	Gas in chamber	Increase of CO ml
1	guinea pig	355	oxygen	0.041
2	» »	415	»	0.007
3	» »	304	»	0.040
	Average	358		0.029
4	rabbit	3,670	oxygen	0.03
5	»	3,510	»	0.13
6	»	3,050	»	0.22
	Average	3,410		0.13
7	rabbit	2,500	air	0.03
8	»	3,670	»	0.01
9	»	2,595	»	0.04
10	»	3,610	»	0.055
	Average	3,094		0.034

Results.

In tables 1 and 2 are recorded the results of the experiments which have been accepted considering the above mentioned conditions. Both in the case of guinea pigs and in rabbits, CO has definitely been demonstrated in the chamber after 12 and 10 hours confinement respectively in the chamber, even when there has been no supply of CO. The CO concentration corresponded on the average to a formation of 0.029 and 0.13 ml CO for guinea pigs and rabbits respectively in oxygen and 0.034 for rabbits in air. After the supply of CO in a concentration of 0.01 % a predominant

Table 2.

Changes in CO volume in an airtight chamber with guinea pigs and rabbits to which CO was administered to different concentrations. Stay in chamber after 2 hours equilibration period 12 and 10 hours respectively for guinea pigs and rabbits.

No.	Animals	Body wt g	Gas in chamber	Approximate CO conc. %	Change in CO volume ml
1	guinea pig	235	oxygen	0.01	+ 0.035
2	» »	290	»	0.01	+ 0.05
3	» »	327	»	0.01	+ 0.04
4	» »	333	»	0.01	— 0.012
	Average	295			+ 0.028
5	guinea pig	343	oxygen	0.05	— 0.100
6	» »	347	»	0.05	— 0.027
7	» »	358	»	0.05	— 0.016
8	» »	361	»	0.05	— 0.013
9	» »	406	»	0.05	— 0.080
10	» »	520	»	0.05	— 0.040
11	» »	354	»	0.05	+ 0.075
	Average	384			— 0.029
12	rabbit	2,905	oxygen	0.01	+ 0.045
13	»	3,422	»	0.01	— 0.010
14	»	3,680	»	0.01	+ 0.005
15	»	3,752	»	0.01	+ 0.005
	Average	3,440			+ 0.011
16	rabbit	2,910	oxygen	0.05	— 0.25
17	»	2,935	»	0.05	— 0.22
	Average	2,922			— 0.23
18	rabbit	2,685	air	0.09	— 0.22
19	»	2,040	»	0.09	— 0.08
20	»	3,320	»	0.09	+ 0.005
21	»	2,905	»	0.09	— 0.15
22	»	2,560	»	0.09	— 0.09
23	»	3,280	»	0.09	— 0.02
24	»	2,160	»	0.09	— 0.09
25	»	2,510	»	0.09	— 0.26
	Average	2,680			— 0.11

number of the experiments show likewise an increase in CO concentration. With a CO concentration of 0.05 % in oxygen rabbits and guinea pigs show definitely a diminution and rabbits likewise with about 0.09 % in air. In the experiments with oxygen, the decrease has reached as much as 0.029 ml CO in guinea pigs and 0.23 ml CO in rabbits for an experimental period of 12 and 10 hours respectively.

The determination of the blood COHb concentration has been undertaken in a couple of guinea pigs in analogous experiments and has been proved to reach 5.6 % of the saturation value with 0.05 % CO in oxygen in the chamber.

Discussion.

The investigations seem to show that if guinea pigs and rabbits are kept in a closed chamber filled with oxygen or air and in which the CO_2 formed by the animals is absorbed by caustic soda, a measurable volume of CO can be demonstrated during 10 to 12 hours stay. Even with an initial concentration of CO of 0.01 %, an increase in the CO concentration in the chamber can be shown under the same conditions. These observations are in agreement with those of SJÖSTRAND who showed that endogenous formation of CO occurs in the animal organism. Analysing the expired air for CO of rabbits under Dial anaesthesia, SJÖSTRAND (1952) found a more rapid formation than that shown by these experiments, that is to say, 0.036 ml per hour. The difference may depend on technical reasons or be connected with different experimental conditions. Under anaesthesia an increase in CO formation may be thought to occur in consequence of a possible increase of the CO_2 and a decrease in the average O_2 saturation of the blood (MALMSTRÖM and SJÖSTRAND 1952). Another difference between the experiments is that the CO concentration in the blood is increased in the present experiment. Thus the increase of the CO volume in the blood of the animals should be included in the calculations. There seems to be a difference between air and oxygen in the rabbit experiments which to some extent can be explained by the larger increase of the CO volume in the blood of the animals in the experiments with air.

With an initial concentration of CO to 0.05 % in oxygen or 0.09 % in air, it has been possible to demonstrate a slighter diminution of the CO volume in the chamber. This is in agreement with Clark's experiment (1950). In the investigation presented, the decrease in CO found is essentially smaller than that which CLARK found in his experiments on turtles and mice. This may be due to differences in method or animals. The decrease reported by CLARK concerning CO concentration in experiments with the chamber but without animals, is essentially larger than that

obtained under the experimental conditions which were used in the present work which is likely to increase the errors in the method.

CO elimination in experiments with living animals can be explained by the fact that CO can be oxidised in the muscle tissue (FENN and COBB 1932) and apparently in the blood too (SJÖSTRAND 1951). In the muscle tissue, however, oxidation of CO has been demonstrated with a CO pressure of quite a different magnitude than that which is reached in *in vivo* experiments. On the other hand earlier investigations on blood *in vitro*, agree, on the whole, with the experiments recounted.

The possible oxidation of CO in the blood seems not to be of such a magnitude that it to any great extent influences the determination of the endogenous CO formation from the expired air CO volume at the low CO concentrations, which can be demonstrated in the blood under normal conditions and which can be related to the liberation of CO in connection with hemoglobin decomposition. However, with higher COHb concentrations it can be expected that CO elimination plays a significant rôle. With 4 to 5 % COHb the rate of formation in incubated blood has thus been found to agree with the rate of elimination. In like manner the condition appears to hold good in the animal organism.

Summary.

Rabbits and guinea pigs have been confined in a closed gas chamber filled with 100 % oxygen or air and fitted with a CO absorber. Determination of the CO concentration in the chamber has been done, on the one hand in the absence of a supply of CO and on the other after CO has been supplied to concentrations of from 0.01 to 0.09 %. Samples have been taken both after an expected equilibrium has ensued between the concentration of CO in the animal organism and the CO pressure in the chamber, and also 10 to 12 hours later. The investigation has shown:

that if CO is not supplied to the chamber filled with oxygen, CO appears after 12 and 10 hours respectively in experiments on guinea pigs and rabbits, corresponding to a formation of 0.029 and 0.13 ml respectively,

that with an initial CO concentration of around 0.01 % CO, there is an increase of CO concentration,

that with a CO concentration of around 0.05 % in oxygen, the concentration of CO diminishes, corresponding on the average to a loss of 0.029 and 0.23 ml for the guinea pig and rabbit respectively during the observation time of respectively 12 and 10 hours, that also with a CO concentration of about 0.09 % in air a decrease can be found in experiments on rabbits.

The results agree with earlier investigations on incubation of blood at 38° C and can be explained by the fact that CO can be formed from the decomposition of hemoglobin but also can be eliminated and possibly oxidised in the blood. With a COHb concentration of 4 to 5 % it seems that *in vivo* as well as *in vitro*, equilibrium occurs between formation and elimination.

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VOL. 31. INDEX.

Fasc. 1. (21. VI. 1954.)

Pag.

Adrenaline and Noradrenaline Output in Urine after Unilateral and Bilateral Adrenalectomy in Man. By U. S. von Euler, C. Franksson and J. Hellström 1

Adrenaline and Noradrenaline Content of Surgically Removed Human Suprarenal Glands. By U. S. von Euler, C. Franksson and J. Hellström 6

Is the Responsiveness to Epinephrine of the Minute Vessels in the Rat Cecal Mesentary Suitable for Measuring Vasodepressor and Vasoexcitor Material? By H. Baden 9

Renal Function in Hypothermia. By A. Bergstrand and G. Sterky 13

Stimulation of Thyroid by Exogenous Thyrotropic Hormone in Young Guinea Pigs Subjected to Subtotal Thymectomy. By L. Gyllensten and A. Olsson 22

The Effect of the Carbonic Anhydrase Inhibitor, Diamox, on Urine Electrolytes in Normal and Adrenalectomized Dogs, and its Possible Relationship to Serum Bicarbonate. By P. O. Madsen 28

On the Behavior of the "Sodium Pump" in Frog Skin at Various Concentrations of Na Ions in the Solution on the Epithelial Side. By H. Linderholm 36

Relationship between Low Values of Alveolar Carbon Monoxide Concentration and Carboxyhemoglobin Percentage in Human Blood. By A. Carlsten, A. Holmgren, K. Linroth, T. Sjöstrand and G. Ström 62

Alimentary Production of Gallstones in Hamsters. III. By F. Christensen and H. Dam 75

Schmerzimpulse bei Temperaturreizen. Von E. Dodt 83

Fasc. 2—3. (18. VII. 1954.)

On the Protracted Action of Histamine Upon the Oxygen Consumption in Rats. By A. N. I. Apajalahti and S. Piha 97

Monosynaptic Corticospinal Activation of Fore Limb Motoneurones in Monkeys (Macaca mulatta). By C. G. Bernhard and E. Bohm 104

Pag.

Catechol Amines and Substance P in the Mammalian Eye. By H. Dunér, U. S. von Euler and B. Pernow 113
A Method for Enzymic Determination of ATP in Tissues. By O. Forsander, B. Rehell and C.-E. Räihä 119
Studies on the Effect of Adrenaline and Noradrenaline on the Duodenal Secretion in Dog. By K. J. V. Hartiala and T. Toivonen 125
Effect of Thermal Stress and Muscular Exercise, With and Without Insulin Hypoglycaemia, on the Body Temperature, Perspiration Rate, and Electrolyte and Lactate Content of Sweat. By J. Hasan, A. Laamanen and M. Niemi 131
Metabolic Responses of Human Subjects to Severe Acute Thermal Stress. By J. Hasan and M. Niemi 137
Rabbit Red Cell Haemolysing Property of Human Plasma. By E. Hirsjärvi 147
The Effect of Thyreoid Hormone on the Mechanogram of the Isolated Rat Auricle. By L. Hirvonen and E. Jalavisto 155
L'influence de la fréquence de changement de l'intensité de la sensation sur la durée de la sensation. Par A. Hormia 163
Patterns in Perceptual Constancy Experiments and the Phantom Limb Phenomenon in Amputees. By E. Jalavisto 167
Observations Concerning the Absorption of Vitamin B_{12} by Cells of Escherichia Coli. By J. Jännes 183
Blood Eosinophil Responses to ACTH in Hyperemesis Gravidarum. By P. A. Järvinen and V. J. Uuspää 187
Permeability of Placenta of the Guinea Pig to Glucose and Fructose. By M. J. Karvonen and N. Räihä 194
The Effect of Certain Choline Esters on the Chemoceptor Activity in the Carotid Body. By G. Liljestrand and Y. Zotterman .. 203
Insulin and Glucose Uptake by the Liver. By E. Lundsgaard .. 215
The Potassium Absorption by Pigeon Blood Cells. A Considerable Potassium Absorption by Pigeon- and Hen Blood Cells is Observed when a Hypertonic Sodium Chloride Solution is Added. By S. L. Ørskov 221
The Effect of Increasing Concentrations of Tween 20 (Polyoxyalkylene Sorbitan Laurate) on the Surface Tension of Plasma. By E. Orma 230
The Assay of Thyroid Preparations Based on Increased Sensitivity to Adrenaline. By P. Peltola and A. Vartiainen 236
Studies on Circulatory, Respiratory and Thermal Adaptation During Heavy Exercise. By R. Ruosteenoja 248
Glucuronic Acid Metabolism in Guinea Pigs. By P. E. Simola .. 263
Rhythmical Potential and Impedance Variations in Isolated Frog Skin Induced by Lithium Ions. By T. Teorell 268
On the Vibrational Sensitivity in Different Regions of the Body Surface. By A. Wilska 285
The Hypothalamus, a Relay Station of the Sympathetic Vasodilator Tract. By S. Eliasson, P. Lindgren and B. Uvnäs 290

Fasc. 4. (20. VIII. 1954.)

Pag.

The Effect of Vitamin D on the Absorption of Inorganic Phosphate. By A. CARLSSON 301

The Cause of Hypophosphatemia and Hypocalcemia in Vitamin D Deficiency. By A. CARLSSON 308

The Influence of Vitamin D on the Uptake of S^{35}-Labelled Sulfate in the Bones. By A. CARLSSON 312

The Effect of Vitamin D on the Citric Acid Metabolism. By A. CARLSSON and G. HOLLUNGER 317

Metabolism of Bone Sodium in Rats Investigated with Na^{22}. By G. C. H. BAUER 334

A Rapid Method for the Simultaneous Determination of Calcium and Sodium in Bone. By G. C. H. BAUER 351

Experimental Studies of the Human Fetus in Prolonged Asphyxia. By G. ENHÖRNING and B. WESTIN 359

Prothrombin Times of Mixtures of Various Types of Plasmas with Lowered Prothrombin Activity. By H. DAM and E. SØNDERGAARD 376

Formation and Elimination of CO in Mammals. By G. METZ and T. SJÖSTRAND 384

Supplementum 112. Distribution and Effects of Catechol Amines in Some Invertebrates and Lower Vertebrates. By E. ÖSTLUND.

Supplementum 113. Effect of N-methylethylphenylbarbituric Acid (Prominal) on the Cholesterol Content in the Ovarian Interstitial Gland of the Rat. By L. CLAESSON.

The Effect of Cholchicine on the Increase in Ovarian Weight and Phospholipids at Gonadotrophic Stimulation. By L. CLAESSON.

Quantitative Relationship between Gonadotrophic Stimulation and Lipid Changes in the Interstitial Gland of the Rabbit Ovary. By L. CLAESSON.

The Intracellular Localization of the Esterified Cholesterol in the Living Interstitial Gland Cell of the Rabbit Ovary. By L. CLAESSON.

The Structure of the Adrenaline and Noradrenaline Containing Granules in the Adrenal Medullary Cells with Reference to the Storage and Release of the Sympathomimetic Amines. By N.-Å. HILLARP and B. NILSON.

Supplementum 114. VIII Scandinavian Physiological Congress. Helsingfors 17th—20th August 1954. Abstracts of Communications.

INDEX AUCTORUM.

Pag.

APAJALAHTI, A. N. I. and S. PIHA, Action of Histamine on Oxygen Consumption 97

BADEN, H., Responsiveness to Epinephrine of the Minute Vessels in the Rat Cecal Mesentery 9

BAUER, G. C. H., Metabolism of Bone Sodium in Rats 334

BAUER, G. C. H., Method for Simultaneous Determination of Calcium and Sodium in Bone 351

BERGSTRAND, A. and G. STERKY, Renal Function in Hypothermia 13

BERNHARD, C. G. and E. BOHM, Activation of Fore Limb Motoneurones in Monkeys 104

BOHM, E. and C. G. BERNHARD, Activation of Fore Limb Motoneurones in Monkeys 104

CARLSSON, A., Vitamin D on Absorption of Inorganic Phosphate 301

CARLSSON, A., Hypophosphatemia and Hypocalcemia in Vitamin D Deficiency 308

CARLSSON, A., Vitamin D on the Uptake of S^{35}-Labelled Sulfate in the Bones 312

CARLSSON, A. and G. HOLLUNGER, Vitamin D on Citric Acid Metabolism 317

CARLSTEN, A., A. HOLMGREN, K. LINROTH, T. SJÖSTRAND and G. STRÖM, Low Values of Alveolar Carbon Monoxide Concentration and Carboxyhemoglobin Percentage in Human Blood 62

CHRISTENSEN, F. and H. DAM, Gallstones in Hamsters. III 75

DAM, H. and F. CHRISTENSEN, Gallstones in Hamsters. III 75

DAM, H. and E. SØNDERGAARD, Prothrombin Times 376

DODT, E., Schmerzimpulse bei Temperaturreizen 83

DUNÉR, H., U. S. VON EULER and B. PERNOW, Catechol Amines and Substance P in Mammalian Eye 113

ELIASSON, S., P. LINDGREN and B. UVNÄS, The Hypothalamus, a Relay Station of Sympathetic Vasodilator Tract 290

ENHÖRNING, G. and B. WESTIN, Human Fetus in Prolonged Asphyxia 359

VON EULER, U. S., C. FRANKSSON and J. HELLSTRÖM, Adrenaline and Noradrenaline Output after Adrenalectomy 1

VON EULER, U. S., C. FRANKSSON and J. HELLSTRÖM, Adrenaline and Noradrenaline Content of Human Suprarenal Glands 6

VON EULER, U. S., H. DUNÉR and B. PERNOW, Catechol Amines and Substance P in Mammalian Eye 113

FORSANDER, O., B. REHELL and C.-E. RÄIHÄ, Enzymic Determination of ATP in Tissues 119

Pag.

FRANKSSON, C., U. S. VON EULER and J. HELLSTRÖM, Adrenaline and Noradrenaline Output after Adrenalectomy 1

FRANKSSON, C., U. S. VON EULER and J. HELLSTRÖM, Adrenaline and Noradrenaline Content of Human Suprarenal Glands 6

GYLLENSTEN, L. and A. OLSSON, Stimulation of Thyroid by Exogenous Thyrotropic Hormone 22

HARTIALA, K. J. V. and T. TOIVONEN, Effect of Adrenaline and Noradrenaline on the Duodenal Secretion 125

HASAN, J., A. LAAMANEN and M. NIEMI, Thermal Stress and Muscular Exercise 131

HASAN, J. and M. NIEMI, Metabolic Responses to Thermal Stress 137

HELLSTRÖM, J., U. S. VON EULER and C. FRANKSSON, Adrenaline and Noradrenaline Output after Adrenalectomy 1

HELLSTRÖM, J., U. S. VON EULER and C. FRANKSSON, Adrenaline and Noradrenaline Content of Human Suprarenal Glands 6

HIRSJÄRVI, E., Rabbit Red Cell Haemolysing Property of Human Plasma 147

HIRVONEN, L. and E. JALAVISTO, Thyreoid Hormone on Mechanogram of Isolated Rat Auricle 155

HOLLUNGER, G. and A. CARLSSON, Vitamin D on Citric Acid Metabolism 317

HOLMGREN, A., A. CARLSTEN, K. LINROTH, T. SJÖSTRAND and G. STRÖM, Low Values of Alveolar Carbon Monoxide Concentration and Carboxyhemoglobin Percentage in Human Blood 62

HORMIA, A., Changement de l'intensité de la sensation et durée de la sensation 163

JALAVISTO, E. and L. HIRVONEN, Thyreoid Hormone on Mechanogram of Isolated Rat Auricle 155

JALAVISTO, E., Phantom Limb Phenomenon in Amputees 167

JÄNNES, J., Absorption of Vitamin B_{12} by Escherichia Coli 183

JÄRVINEN, P. A. and V. J. UUSPÄÄ, Responses to ACTH in Hyperemesis Gravidarum 187

KARVONEN, M. J. and N. RÄIHÄ, Permeability of Placenta to Glucose and Fructose 194

LAAMANEN, A., J. HASAN and M. NIEMI, Thermal Stress and Muscular Exercise 131

LILJESTRAND, G. and Y. ZOTTERMAN, Choline Esters on Chemoceptor Activity in the Carotid Body 203

LINDERHOLM, H., "Sodium Pump" in Frog Skin 36

LINDGREN, P., S. ELIASSON and B. UVNÄS, The Hypothalamus, a Relay Station of Sympathetic Vasodilator Tract 290

LINROTH, K., A. CARLSTEN, A. HOLMGREN, T. SJÖSTRAND and G. STRÖM, Low Values of Alveolar Carbon Monoxide Concentration and Carboxyhemoglobin Percentage in Human Blood 62

LUNDSGAARD, E., Insulin and Glucose Uptake by the Liver 215

MADSEN, P. O., Effect of the Carbonic Anhydrase Inhibitor, Diamox, on Urine Electrolytes 28

Pag.

METZ, G. and T. SJÖSTRAND, Formation and Elimination of CO in Mammals 384
NIEMI, M., J. HASAN and A. LAAMANEN, Thermal Stress and Muscular Exercise 131
NIEMI, M. and J. HASAN, Metabolic Responses to Thermal Stress 137
OLSSON, A. and L. GYLLENSTEN, Stimulation of Thyroid by Exogenous Thyrotropic Hormone 22
ORMA, E., Effect of Tween 20 on Surface Tension of Plasma 230
ØRSKOV, S. L., Potassium Absorption by Pigeon Blood Cells 221
PELTOLA, P. and A. VARTIAINEN, Assay of Thyroid Preparations 236
PERNOW, B., H. DUNÉR and U. S. VON EULER, Catechol Amines and Substance P in Mammalian Eye 113
PIHA, S., and A. N. I. APAJALAHTI, Action of Histamine on Oxygen Consumption 97
REHELL, B., O. FORSANDER and C.-E. RÄIHÄ, Enzymic Determination of ATP in Tissues 119
RUOSTEENOJA, R., Studies on Circulatory Adaptation During Heavy Exercise 248
RÄIHÄ, C.-E., O. FORSANDER and B. REHELL, Enzymic Determination of ATP in Tissues 119
RÄIHÄ, N. and M. J. KARVONEN, Permeability of Placenta to Glucose and Fructose 194
SIMOLA, P. E., Glucuronic Acid Metabolism 263
SJÖSTRAND, T., A. CARLSTEN, A. HOLMGREN, K. LINROTH and G. STRÖM, Low Values of Alveolar Carbon Monoxide Concentration and Carboxyhemoglobin Percentage in Human Blood 62
SJÖSTRAND, T. and G. METZ, Formation and Elimination of CO in Mammals 384
STERKY, G. and A. BERGSTRAND, Renal Function in Hypothermia 13
STRÖM, G., A. CARLSTEN, A. HOLMGREN, K. LINROTH and T. SJÖSTRAND, Low Values of Alveolar Carbon Monoxide Concentration and Carboxyhemoglobin Percentage in Human Blood ... 62
SØNDERGAARD, E. and H. DAM, Prothrombin Times 376
TEORELL, T., Variations in Isolated Frog Skin Induced by Lithium Ions 268
TOIVONEN, T. and K. J. V. HARTIALA, Effect of Adrenaline and Noradrenaline on the Duodenal Secretion 125
UUSPÄÄ, V. J. and P. A. JÄRVINEN, Responses to ACTH in Hyperemesis Gravidarum 187
UVNÄS, B., S. ELIASSON and P. LINDGREN, The Hypothalamus, a Relay Station of Sympathetic Vasodilator Tract 290
VARTIAINEN, A. and P. PELTOLA, Assay of Thyroid Preparations 236
WESTIN, B. and G. ENHÖRNING, Human Fetus in Prolonged Asphyxia 359
WILSKA, A., Vibrational Sensitivity of the Body Surface 285
ZOTTERMAN, Y. and G. LILJESTRAND, Choline Esters on Chemoceptor Activity in the Carotid Body 203

Pag.
n
, 384
s-
. 131
ss 137
r-
. 22
. 230
. 221
as 236
es
. 113
n
, 97
s-
. 119
g
, 248
a-
. 119
o
, 194
. 263
f.
n
, 62
n
. 384
a 13
-
-
, 62
. 376
n
, 268
-
. 125
-
. 187
,
. 290
s 236
-
. 359
. 285
-
. 203